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INHIBITION OF RABBIT AORTIC ANGIOTENSIN II (AII) RECEPTOR BY CV-11974, A NEW NONPEPTIDE AII ANTAGONIST

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Abstract—The angiotensin II (AII) antagonistic action of CV-11974 (2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]benzimidazole-7-carboxylic acid) was investigated in an AII-receptor binding assay using rabbit aortic membranes and an AII-induced contraction assay using rabbit aortic strips. A single class of [^{125}I]AII-(Sar^1,Ile^8) binding sites was found in the membranes with a dissociation constant (K_d) of 0.15 nM and a receptor concentration (B_{max}) of 86.9 fmol/mg protein. CV-11974 markedly reduced K_d without affecting B_{max}. The specific binding of [^{125}I]AII-(Sar^1,Ile^8) in this preparation was inhibited competely by CV-11974 [the inhibition constant (K_i) = 0.64 nM], DuP 753 [an angiotensin II type I (AT_1) receptor-selective antagonist] (K_i = 51 nM) and EXP3174 (an active metabolite of DuP 753) (K_i = 6.8 nM), but was not affected by PD123177 (an AT_2 receptor-selective antagonist). These results suggest that the single binding site in rabbit aortic membranes is an AT_1 receptor subtype. The affinity of CV-11974 to these AT_1 receptors was approximately 80 and 10 times higher than that of DuP 753 and EXP3174, respectively. CV-11974 showed no appreciable affinity for the AT_2 receptors found in bovine cerebellum. In the *in vitro* functional study, CV-11974 markedly reduced the AII-induced maximal contractile response of rabbit aortic strips (pD'_2 = 9.97). In contrast, Compound 7-H, which lacks the carboxyl group at the benzimidazole ring of CV-11974, inhibited the contraction in a competitive manner. The inhibition by CV-11974 was long lasting. These results suggest that CV-11974 is a potent and long-acting AT_1 receptor-selective, competitive antagonist. The carboxyl group at the benzimidazole ring plays an important role in the interaction between CV-11974 and the AT_1 receptor.

Angiotensin II (AII‡) has important roles in the regulation of blood pressure and fluid volume homeostasis. AII produces its physiological effects via binding to specific receptors in the plasma membranes of various tissues. Recent binding studies using several non-peptide AII receptor antagonists have confirmed the existence of at least two AII receptor subtypes [1, 2]. The AII receptor subtype having a high affinity for DuP 753 has been classified as an AT_1 receptor and the subtype having a high affinity for PD123177 or CGP42112A as an AT_2 receptor [3]. The AT_1 receptor subtype has been found in tissues such as vascular smooth muscle [4], liver [5], kidney [6] and rat adrenal cortex [2], whereas the AT_2 subtype has been identified in tissues such as bovine cerebellum [7], uterus [4], canine pancreas [8] and rat adrenal medulla [2]. As far as a biological function is concerned, it has been shown that the AT_1 receptor subtype is associated with the regulation of blood pressure; this subtype is responsible for vascular contraction [9] and DuP 753 decreases blood pressure in renin-dependent hypertensive rats [10] and spontaneously hypertensive rats [11] *in vivo*. However, the function for the AT_2 receptor subtype *in vivo* is still unknown.

The purpose of the present study was to investigate the *in vitro* pharmacological properties of the AII antagonist CV-11974 [12], and to determine the mode of inhibition by this antagonist in two *in vitro* assays.

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‡ Abbreviations: AI, angiotensin I; AII, angiotensin II; AIII, angiotensin III; A(1–7), the N-terminal heptapeptide angiotensin$^{1-7}$; AII-(Sar^1,Ile^8), sarcosine^1, isoleucine^8-angiotensin II; AT_1, angiotensin II type 1 receptor; AT_2, angiotensin II type 2 receptor; AVP, arginine vasopressin; DMSO, dimethyl sulfoxide; ET, endothelin; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; and PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Materials. [^{125}I]Sarcosine^1, isoleucine^8-angiotensin II [AII-(Sar^1,Ile^8)] (2200 Ci/mmol) and an AT_2 receptor binding kit (Drug Discovery Systems: angiotensin II receptor type 2, NED-001) were purchased from New England Nuclear (U.S.A.) and Amersham (U.S.A.), respectively. AI, AII, AIII, AII-(Sar^1,Ile^8), A(1–7), arginine vasopressin (AVP) and endothelin (ET) were purchased from Peptide

Institute, Inc. (Japan). Norepinephrine (NE) and 5-hydroxytryptamine (5-HT) were purchased from the Sigma Chemical Co. (U.S.A.). Compound 7-COOH (CV-11974), Compound 4-COOH (2-ethoxy-1-[[2'-(1*H* - tetrazol - 5 - yl)biphenyl - 4 - yl]methyl]-benzimidazole-4-carboxylic acid), Compound 5-COOH (2 - ethoxy - 1 - [[2' - (1*H* - tetrazol - 5 - yl)biphenyl-4-yl]methyl]benzimidazole-5-carboxylic acid), Compound 6-COOH (2-ethoxy-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]benzimidazole-6-carboxylic acid), Compound 7-H (2-ethoxy-1-[[2' - (1*H* - tetrazol - 5 - yl)biphenyl - 4 - yl]methyl]-benzimidazole), TCV-116 ((±)-1-(cyclohexyl-oxycarbonyloxy)ethyl - 2 - ethoxy - 1 - [[2' - (1*H* - tetrazol-5-yl)biphenyl-4-yl]methyl]benzimidazole-7-carboxylate), DuP 753, EXP3174 [13] and PD123117 were synthesized at Pharmaceutical Research Laboratories I of Takeda Chemical Industries (Japan). Bovine serum albumin bovine albumin fraction V fatty acid poor) was obtained from Miles Inc. (U.S.A.). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and other chemicals were obtained from Wako Pure Chemical Industries (Japan). A protein assay kit was purchased from Bio-Rad Laboratories (U.S.A.). The non-peptide antagonists were dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and diluted to the desired concentration with the buffer used in the receptor binding assays and in the contractile assay using aortic strips.

Preparation of rabbit aortic membranes. The membranes were prepared according to the method of Chang *et al.* [14] and Chang and Lotti [15] with slight modifications. The aorta was quickly removed from male rabbits (Kitayama breeding system: Japanese White; 3–4 kg) and cleaned. The aorta was minced and then homogenized twice with a polytron (Brinkmann, Switzerland) (maximal speed for 30 sec) in approximately 12 times the volume of buffer [0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM Tris–HCl, pH 7.4]. The homogenate was centrifuged at 1,500 *g* for 10 min at 4°, and the supernatant was centrifuged again as before. The supernatant was centrifuged at 105,000 *g* for 30 min at 4°. The pellet was resuspended in the buffer (10 mM $MgCl_2$, 1 mM PMSF, 5 mM EDTA-2Na and 100 mM Tris–HCl, pH 7.4). The membranes were stored at −80° until used. Protein was determined by the dye staining technique of Bradford [16]. Bovine serum albumin was used as the standard.

Rabbit aortic binding assay. The experiments were done according to the method of Chang *et al.* [14] and Chang and Lotti [15] with slight modifications. The membranes (25 μg/tube) were incubated with 0.02 to 3.0 nM [^{125}I]AII-(Sar^1,Ile^8) for Scatchard analysis or 0.8 nM radiolabeled AII-(Sar^1,Ile^8) for displacement studies, with various concentrations of non-peptide antagonists, angiotensin peptides and vehicle for 90 min at 37°, in a total volume of 200 μL binding buffer (10 mM $MgCl_2$, 5 mM EDTA-2Na, 1 mM PMSF, 0.25% bovine serum albumin and 100 mM Tris–HCl, pH 7.4). The reaction was terminated by rapid filtration of the incubation solution through glass fiber filters (Whatman GF/B filters) presoaked with 1% polyethylenimine in saline. The filters were washed immediately 3 times with 3 mL of ice-cold saline. The radioactivity trapped on the filter was determined by a γ-counter (ALOKA, ARC-1000, Japan). The specific binding was defined as the difference between the total binding and the non-specific binding in the presence of 10 μM unlabeled AII-(Sar^1,Ile^8). In this study, we used [^{125}I]AII-(Sar^1,Ile^8) as the radioligand being more resistant than AII to proteolytic cleavage [17]. Under this condition, the total binding was 2.0% of the used radiolabeled ligand and the non-specific binding was 28.1% of the total binding.

Cerebellar binding assay. Displacement studies of [^{125}I]AII (2200 Ci/mmol) binding to the AT_2 receptor by non-peptide antagonists and angiotensin peptides were performed using a receptor binding kit. The membranes (12.5 mg/mL tissue wet weight), containing a single population of AII binding sites classified as AT_2 receptors [7], prepared from bovine cerebellum were incubated with [^{125}I]AII (0.1 nM) in a total volume of 400 μL binding buffer for 60 min at 37°. Non-specific binding was determined in the presence of 1 μM unlabeled AII. Other experiments were done as described above.

AII-induced contraction of rabbit aortic strips. Helical strips of the isolated aorta from male rabbits (Kitayama breeding system: Japanese White; 3–4 kg) were mounted on tissue holders under an initial tension of 2.0 g in organ baths containing 20 mL Krebs–Henseleit solution (120 mM NaCl, 4.7 mM KCl, 4.7 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose). The organ baths were kept at 37° and gassed continuously with 95% O_2–5% CO_2. The tissues were permitted to be equilibrated for 2–3 hr before testing. Contractile tension was recorded on a recorder (San-ei, RECTI-HORIZ-8K, Japan) via a force displacement transducer (MINEBEA, UL-10GR, Japan) and a preamplifer (San-ei, 6M52, Japan). AII was added cumulatively to the organ baths. After washing the tissues for 2 hr, various concentrations of antagonists were added and incubated with the tissues for at least 30 min. A cumulative concentration–response curve for AII in the presence of each antagonist was determined again as before. The result at each concentration was expressed as a percentage of maximum response to AII obtained in the first curve. The pA_2 and pD'_2 values of each antagonist were calculated according to the method of van Rossum [18]. To determine the selectivity of CV-11974, the effect of CV-11974 on the NE (10^{-6} M); 5-HT (10^{-6} M); ET (10^{-8} M); $PGF_{2\alpha}$ (2×10^{-6} M)- and KCl (60 mM)-induced contraction was also checked.

Data analysis. The inhibition constant (K_i) values were calculated from the respective IC_{50} (the concentration of the drug required for 50% inhibition) values using the following formula: $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of radiolabeled ligand and K_d is its dissociation constant obtained from Scatchard analysis. The IC_{50} values were determined by regression analysis, using the RS/1 (BBN Software Products Corp., Cambridge, MA).

Statistics. All experiments were performed in duplicate and repeated three to four times. All data are given as means ± SEM. Differences were

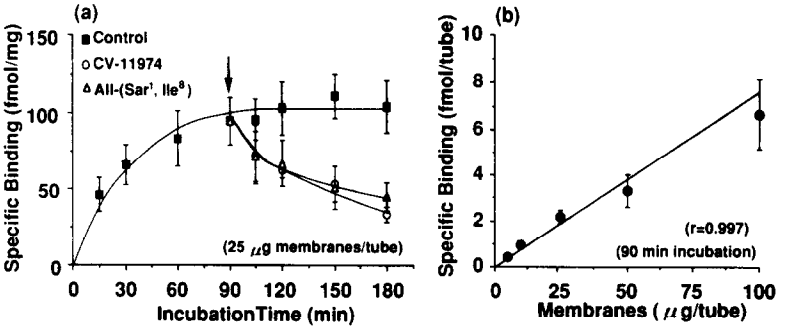

Fig. 1. (a) Association and dissociation of [^{125}I]AII-(Sar^1,Ile^8) to rabbit aortic membranes. Dissociation was initiated by addition of unlabeled AII-(Sar^1,Ile^8) and CV-11974 (10 μM) at 90 min (arrow) after incubation. (b) Relationship between specific binding and protein content in rabbit aortic membranes. Data are means ± SEM (N = 3).

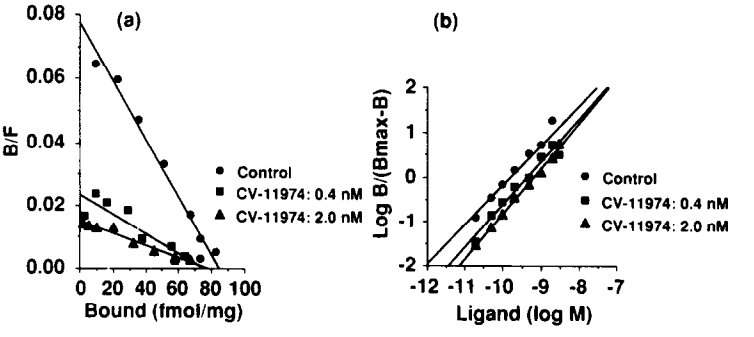

Fig. 2. (a) Scatchard plot of [^{125}I]AII-(Sar^1,Ile^8) binding to rabbit aortic membranes. The K_d values for control, 0.4 nM CV-11974 and 2.0 nM CV-11974 groups were 0.15, 0.40 and 0.66 nM, respectively. The B_{max} values were 86.9, 75.5 and 78.6 fmol/mg protein, respectively. (b) Hill-plot analysis. The Hill coefficients for control, 0.4 nM CV-11974 and 2.0 nM CV-11974 groups were 0.88, 0.95 and 1.01, respectively. The results for control are the means of four experiments and the results in the presence of CV-11974 are the means of two typical experiments. B/F: bound/free.

considered statistically significant at $P < 0.05$, using Student's unpaired *t*-test.

RESULTS

Binding of [^{125}I]*AII-(Sar^1,Ile^8) to rabbit aorta.* At a concentration of 25 μg of membranes/tube, the specific binding of [^{125}I]AII-(Sar^1,Ile^8) (0.8 nM) to the membranes reached a steady-state level after 90 min of incubation, and was displaced by excess unlabeled AII-(Sar^1,Ile^8) (10 μM) and CV-11974 (10 μM) (Fig. 1a). The specific binding was linear from 5 to 100 μg of membranes/tube for a 90-min incubation (Fig. 1b).

Figure 2 shows Scatchard plots (a) and Hill plots (b) of the specific binding in the absence and presence of CV-11974. The control K_d and B_{max} values determined in this preparation were 0.15 ± 0.02 nM (N = 4) and 86.9 ± 4.4 fmol/mg protein (N = 4), respectively. [^{125}I]AII-(Sar^1,Ile^8) interacted with a single population of binding sites. CV-11974 (0.4 and 2.0 nM) competitively interacted with this binding site. At the concentration tested, CV-11974 increased the K_d value markedly without affecting the B_{max} value. The control Hill coefficient was not different from unity, and CV-11974 did not affect the slope.

In displacement studies using rabbit aortic membranes, the affinity of non-peptide antagonists and angiotensin peptides was determined (Fig. 3). All non-peptide and peptide antagonists except for PD123177 and AVP inhibited the specific binding of [^{125}I]AII-(Sar^1,Ile^8) in a concentration-dependent, monophasic manner. The data indicate that the single binding site in rabbit aortic membranes is of the AT_1 receptor subtype. The affinity of CV-11974 to this AT_1 receptor was approximately 80 and 10 times higher than that of DuP 753 and EXP3174, respectively (Table 1), and also higher than that of AII. CV-11974 was approximately 250 times more potent than its prodrug, TCV-116. Additionally, all

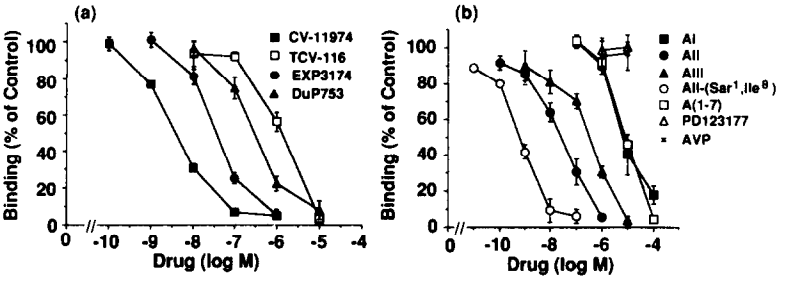

Fig. 3. Displacement of the specific binding of [^{125}I]AII-(Sar^1,Ile^8) to rabbit aortic membranes by (a) CV-11974, TCV-116, EXP3174 and DuP 753 and (b) angiotensin peptides, PD123177 and AVP. Control values are given in Table 1. Data are means ± SEM (N = 3–4).

Table 1. Inhibitory effects of non-peptide AII receptor antagonists, angiotensin peptides and AVP on the specific binding of [^{125}I]AII-(Sar^1,Ile^8) or [^{125}I]AII to rabbit aortic or bovine cerebellar membranes, respectively

	AT_1 Rabbit aorta K_i (M)	AT_2 Bovine cerebellum K_i (M)
CV-11974	$6.40 \pm 0.43 \times 10^{-10}$	$>10^{-5}$
TCV-116	$1.67 \pm 0.12 \times 10^{-7}$	ND
EXP3174	$6.76 \pm 1.32 \times 10^{-9}$	$>10^{-5}$
DuP 753	$5.10 \pm 1.10 \times 10^{-8}$	$>10^{-5}$
AI	$1.87 \pm 0.10 \times 10^{-6}$	$3.05 \pm 0.11 \times 10^{-7}$
AII	$4.52 \pm 2.28 \times 10^{-9}$	$1.01 \pm 0.39 \times 10^{-10}$
AIII	$4.73 \pm 1.11 \times 10^{-8}$	$8.71 \pm 2.35 \times 10^{-11}$
AII(Sar^1,Ile^8)	$1.08 \pm 0.21 \times 10^{-10}$	ND
A(1–7)	$1.38 \pm 0.14 \times 10^{-6}$	$5.18 \pm 0.11 \times 10^{-7}$
PD123177	$>10^{-4}$	$2.38 \pm 0.32 \times 10^{-7}$
AVP	$>10^{-4}$	$>10^{-4}$
7-H	$5.97 \pm 1.01 \times 10^{-8}$	ND
4-COOH	$9.91 \pm 2.80 \times 10^{-7}$	ND
5-COOH	$1.24 \pm 0.25 \times 10^{-6}$	ND
6-COOH	$6.77 \pm 0.49 \times 10^{-8}$	ND

Data are means ± SEM (N = 3–4). ND: not determined.

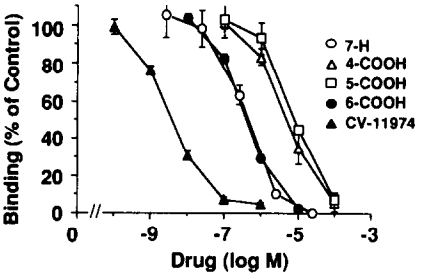

Fig. 5. Displacement of the specific binding of [^{125}I]AII-(Sar^1,Ile^8) to rabbit aortic membranes by CV-11974 isomers and Compound 7-H. Control values are given in Table 1. Data are means ± SEM (N = 3–4).

angiotensin peptides, including A(1–7) inhibited the specific binding in a concentration-dependent manner (Fig. 3b).

Binding of [^{125}I]*AII to bovine cerebellum.* In displacement studies for AT_2 receptors, the affinity of the compounds and angiotensin peptides was determined (Fig. 4). PD123177 and all angiotensin peptides completely inhibited the specific binding of

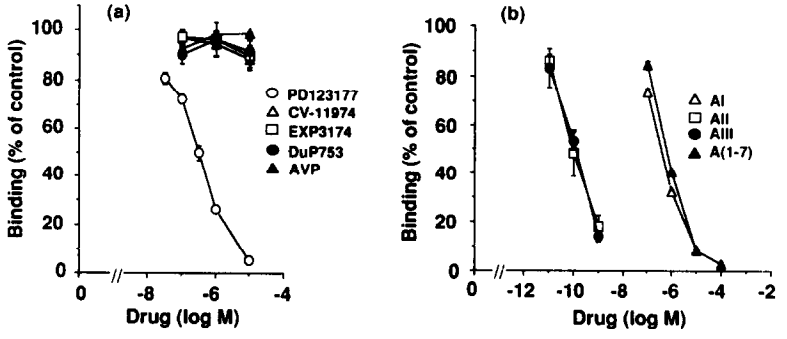

Fig. 4. Displacement of the specific binding of [^{125}I]AII to bovine cerebellar membranes by (a) PD123177, CV-11974, EXP3174, DuP 753 and AVP and (b) angiotensin peptides. Control values are given in Table 1. Data are means ± SEM (N = 3).

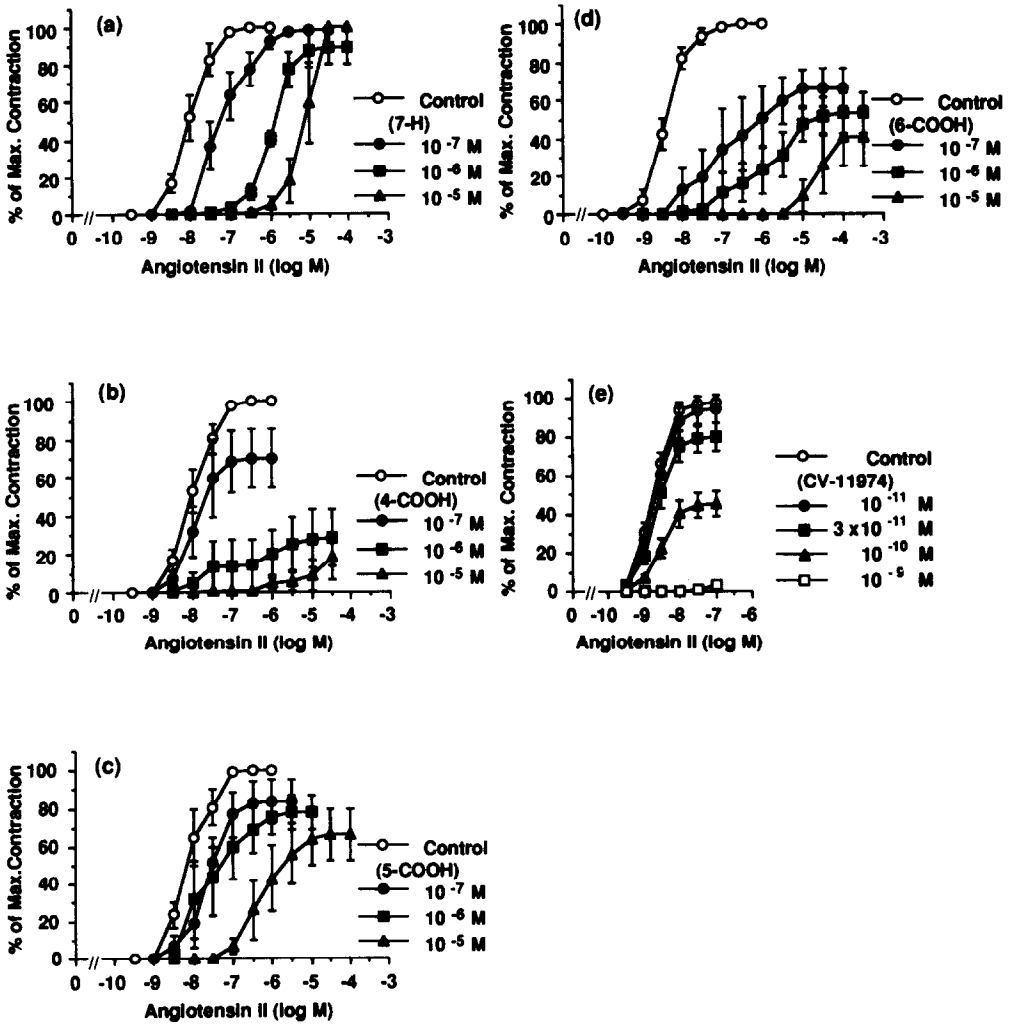

Fig. 6. Inhibitory effects of CV-11974 isomers and Compound 7-H on the AII-induced contraction of the rabbit aorta. Maximal contractile response for the control in each experiment: (a) 0.82 ± 0.22 g (N = 7), (b) 0.84 ± 0.20 g (N = 8), (c) 0.72 ± 0.14 g (N = 6), (d) 0.88 ± 0.19 g (N = 8), and (e) 1.46 ± 0.12 g (N = 7). The results are summarized in Table 2. Data are means ± SEM (N = 6–8).

[^{125}I]AII to bovine cerebellum in a concentration-dependent manner. However, CV-11974, TCV-116, DuP 753, EXP3174 and AVP did not reduce the specific binding at all.

Effects of isomers of CV-11974 on [^{125}I]AII-(Sar^1,Ile^8) binding to rabbit aorta. In displacement studies for AT_1 receptors, the affinity of the isomers of CV-11974 was determined (Fig. 5). CV-11974 showed the highest affinity to the AT_1 receptor in rabbit aortic membranes.

Effects of CV-11974 on AII-induced contraction in rabbit aorta. CV-11974 reduced the maximal contraction without an apparent shift to the right of the concentration–contractile response curve (Fig. 6). The antagonism by CV-11974 was dependent on the incubation period of the aorta with CV-11974; the inhibition by CV-11974 (10^{-10} M) was 44 ± 5% (N = 3), 65 ± 3% (N = 5) and 88 ± 5% (N = 3) in the case of 30-, 60- and 90-min pretreatments, respectively. The inhibition of the AII (10^{-8} M)-induced contraction by pretreatment with CV-11974 (3×10^{-10} M) for 30 min (53 ± 9% inhibition, N = 6 was not recovered at 2 hr after washing (55 ± 7% inhibition, N = 6). In contrast, the inhibition by pretreatment with EXP3174 (10^{-9} M) for 30 min (70 ± 5% inhibition, N = 6) was recovered at 2 hr after washing (37 ± 4% inhibition, N = 6, $P < 0.05$ vs the inhibition before washing). However, the inhibitory effect of CV-11974 was not irreversible because the almost complete inhibition by the pretreatment (10^{-9} M) for 30 min (97 ± 0.2% inhibition, N = 5) was recovered to some degree at 6 hr after washing (41 ± 7% inhibition, N = 5, $P < 0.05$ vs the inhibiton before washing). These results indicate that the inhibitory effect of CV-11974 is not irreversible, but the duration of the inhibitory effect of CV-11974 is longer than that of EXP3174.

Table 2. Inhibitory effects of CV-11974 isomers and Compound 7-H on the AII-induced contraction of the rabbit aorta

Compound	pA_2	pD'_2	Mode
7-H	7.93 ± 0.21	ND	Competitive
4-COOH	ND	6.43 ± 0.34	Non-competitive
5-COOH	6.85 ± 0.13	5.21 ± 0.27	Mix
6-COOH	8.54 ± 0.82	5.99 ± 0.31	Mix
CV-11974	ND	9.97 ± 0.10	Non-competitive

Data are means ± SEM (N = 4–7). ND: not detected.

CV-11974 (10^{-5} M) did not affect the contraction induced by 5-HT (10^{-6} M), NE (10^{-6} M), ET (10^{-8} M), $PGF_{2\alpha}$ (2×10^{-6} M) and KCl (60 mM) (data not shown).

Effects of isomers of CV-11974 on AII-induced contraction in rabbit aorta. The nature of AII antagonism of the isomers of CV-11974 was investigated in AII-induced rabbit aortic contraction as a functional assay (Fig. 6 and Table 2). Compound 7-H (without a carboxyl group) shifted the concentration–contractile response curve for AII to the right in a parallel manner without a reduction of the maximal contraction (Fig. 6a). The other isomers of CV-11974 except for Compound 7-H reduced AII-induced maximal contraction. Compound 4-COOH and CV-11974, possessing a carboxyl group at the 4- and 7-position, respectively, reduced the maximal contraction without an apparent shift to the right of the concentration–contractile response curve (Fig. 6, b and e). Compound 5-COOH and Compound 6-COOH, possessing a carboxyl group at the 5- and 6-position, respectively, reduced the maximal response and shifted the concentration–contractile response curve for AII to the right (Fig. 6, c and d). In this rabbit aorta contractile assay, the inhibitory potency of CV-11974 ($pD'_2 = 9.97$) was comparable to its binding affinity to rabbit aortic membranes ($K_i = 6.40 \times 10^{-10}$ M) obtained from radioligand displacement studies. A(1–7) did not contract the aortic strips (data not shown), but inhibited the AII-induced contractile response ($pA_2 = 5.81$, $pD'_2 = 4.08$; N = 3) in contrast to AI and AIII.

DISCUSSION

In this report, we have characterized CV-11974 as a new highly potent AII receptor antagonist in a binding assay (rabbit aortic membranes) and a functional assay (contraction of rabbit aorta). In our studies, the AT_1 receptor-selective antagonists, DuP 753 and EXP3174 [13, 19], bound to the membranes and inhibited the specific binding of [^{125}I]AII-(Sar^1,Ile^8) in a monophasic manner with K_i values of 51 and 6.8 mM, respectively. In contrast, the AT_2 receptor-selective antagonist, PD123177 [3], did not bind to the membranes. The potency of EXP3174 was approximately 10 times greater than that of DuP 753 in this preparation. The binding affinities of DuP 753 and EXP3174 to rabbit aortic membranes and the difference of the potency between these two compounds reported here are similar to those previously documented [20, 21] as are the K_d and the B_{max} values for [^{125}I]AII-(Sar^1,Ile^8) [21]. It has been established that there are at least two AII receptor subtypes, AT_1 and AT_2, present in various tissues. Our data show that the subtype in rabbit aortic membranes is an AT_1 receptor subtype. CV-11974 interacted with this receptor without negative or positive cooperation action since the Hill coefficient in the presence of CV-11974 was not different from unity. CV-11974 completely inhibited the specific binding to this AT_1 receptor subtype in a concentration-dependent, monophasic manner with a K_i value of 0.64 nM. CV-11974 had the highest affinity to rabbit aortic membranes of all the compounds and agents used in this report. In addition, the K_i value of CV-11974 is similar to that of L-158,809 (an AT_1 selective antagonist), recently reported by Chang *et al.* [21]. This study demonstrates that the carboxyl group at the 7-position of the benzimidazole ring is very important for binding affinity.

To determine whether CV-11974 interacts reversibly or irreversibly with AII receptors, Scatchard analysis in the absence and presence of CV-11974 was performed. CV-11974 changed the K_d of [^{125}I]-AII-(Sar^1,Ile^8) to the membranes in a concentration-dependent manner without altering the B_{max}. This result suggests that CV-11974 binds to the receptor in a reversible and competitive manner.

In the AII-induced rabbit aorta contraction assay, CV-11974 markedly reduced the maximal response without a shift of the concentration–response curve to the right (Fig. 6e). Such a pattern of antagonism can be observed with competitive antagonists, which are irreversibly or pseudoirreversibly associated with receptors, as well as with non-competitive antagonists [22]. EXP3174 and L-158,809 inhibited the AII-induced contraction of the rabbit aorta in a non-competitive manner [13, 21]. However, L-158,809 antagonized the AII binding in the rabbit aorta in a competitive manner. In these reports, the authors suggested that this pseudoirreversible antagonism might be produced by a slow dissociation of the antagonist–receptor complex [21, 23]. The duration of the antagonistic effect of CV-11974 on the AII-induced contraction was very long. Therefore, CV-11974 may also show pseudoirreversible antagonism due to a slow dissociation from the receptors.

All CV-11974 isomers, possessing a carboxyl group at the benzimidazole ring, inhibited the AII-induced contractile response in a non-competitive manner (Fig. 6b–d). In contrast, Compound 7-H, lacking a carboxyl group, shifted the concentration–response curve to the right in a competitive manner without reducing the maximal response (Fig. 6a). These findings suggest that the carboxyl group plays a key role in determining the receptor–antagonist interaction. A similar hypothesis has been proposed by Wienen *et al.* [23]. DuP 753, which does not have a carboxyl group, antagonizes the AII-induced contractile response of the rabbit aorta in a purely competitive manner [19], but EXP3174, which has a carboxyl group, antagonizes the response in a non-competitive manner [13].

CV-11974 is a selective antagonist of the AII receptor, because CV-11974 did not inhibit the contraction induced by 5-HT, NE, ET, $PGF_{2\alpha}$ and KCl, although we did not investigate whether CV-11974 affects GTP-binding protein.

The longer duration of the inhibitory effects by CV-11974 in the AII-induced contraction assay may be related to the longer duration of the AII antagonism by CV-11974 or TCV-116 than that of EXP3174 or DuP 753 in an *in vivo* system, which has been reported by Shibouta *et al.* [12].

In the AII-binding assay using rabbit aorta and bovine cerebellum, we found that AI and AIII bound to the same binding site as AII (Figs. 3 and 4, and Table 1). These results support the evidence that AII, AI and AIII produce their pharmacological effects via binding to the same receptor [24, 25]. A(1–7) also inhibited the specific binding of [^{125}I]-AII-(Sar^1,Ile^8) to AT_1 receptors in rabbit aortic membranes and the specific binding of [^{125}I]AII to AT_2 receptors in bovine cerebellar membranes (Figs. 3 and 4, Table 1). Moreover, A(1–7) antagonized the AII-induced contraction of the rabbit aorta. Previous studies have indicated that A(1–7) is generated from AI via an ACE-independent pathway in rats [26], dogs [27], and cultured vascular endothelial cells [28]. Furthermore, some recent studies have provided evidence that A(1–7) stimulates the release of prostaglandin and prostacyclin [29]. Interestingly, Ferrario and coworkers [30] reported that in spontaneously hypertensive rats, plasma levels of A(1–7) were greater than those in Wistar–Kyoto rats. Those findings may be related to our results that A(1–7) modulates the vasoconstrictor effect of AII on vascular smooth muscle, although the required concentration may be higher than *in vivo*.

In conclusion, the present study demonstrated that a novel benzimidazole carboxylic acid, CV-11974, is a powerful AT_1 receptor-selective antagonist in rabbit aortic membranes and also showed the potent and long-lasting inhibitory effect on the AII-induced contraction of rabbit aortic strips. Furthermore, it is suggested that the carboxyl group at the 7-position of the benzimidazole ring may play an important role in determining the interaction with the AT_1 receptor. CV-11974 may become a useful tool in the research of the physiological and pharmacological role of AII in cardiovascular diseases such as hypertension and heart failure.

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