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Candesartan (CV-11974) dissociates slowly from the angiotensin AT_1 receptor

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Abstract

The mechanisms of the insurmountable antagonism of 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid, candesartan (CV-11974), an angiotensin AT_1 receptor antagonist, on angiotensin II-induced rabbit aortic contraction were examined in contraction and binding studies. Preincubation of the rabbit aorta with CV-11974 (0.1 nM) for 30 min reduced the maximal contractile response to angiotensin II by approximately 50%. This insurmountable antagonism of CV-11974 was reversed in the presence of losartan (1 μM), a surmountable angiotensin AT_1 receptor antagonist. The inhibitory effect of CV-11974 on angiotensin II-induced contraction persisted longer after washing than did that of losartan but was not irreversible. Scatchard analysis of [^{3}H]CV-11974 binding in bovine adrenal cortical membranes indicated the existence of a single class of binding sites ($K_d = 7.4$ nM). Competition binding studies using angiotensin II receptor agonists and antagonists have demonstrated that [^{3}H]CV-11974 binding sites may be identical to angiotensin AT_1 receptors. The dissociation rate of [^{3}H]CV-11974 binding ($t_{1/2} = 66$ min) was 5 times slower than that of [^{125}I]angiotensin II binding ($t_{1/2} = 12$ min). These results suggest that the insurmountable antagonism by CV-11974 is due to its slow dissociation from angiotensin AT_1 receptors.

Keywords: Candesartan (CV-11974); [^{3}H]CV-11974; Losartan; Angiotensin AT_1 receptor; Association rate constant; Dissociation rate constant

1. Introduction

TCV-116 (candesartan cilexetil), (±)-1-(cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate, is an orally active, potent and long-acting non-peptide angiotensin II receptor antagonist (Kubo et al., 1993). The hypotensive effects of TCV-116 have been observed not only in two-kidney, one-clip hypertensive rats with high plasma renin levels but also in spontaneously hypertensive rats and one-kidney, one-clip hypertensive rats with normal or low plasma renin levels (Wada et al., 1992; Inada et al., 1994). The clinical effectiveness of TCV-116 has also been demonstrated (Ogihara et al., 1994).

Recently, definitive evidence for two distinct subtypes of angiotensin II receptors has been obtained (Chiu et al., 1989; Whitebread et al., 1989), and these have been designated angiotensin AT_1 and AT_2 receptors (Bumpus et al., 1991). It is well established that vascular and adrenal cortical angiotensin II receptors in various species (rat, rabbit, monkey, bovine, etc.) are the angiotensin AT_1 subtype (De Gasparo et al., 1990; Chang and Lotti, 1991; Shibouta et al., 1993) and that major physiological and functional responses to angiotensin II are mediated via angiotensin AT_1 receptors (Wong et al., 1990). In contrast, no clear physiological function for angiotensin AT_2 receptors has yet been identified.

TCV-116 is converted to an active metabolite, CV-11974 (candesartan), 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid, by deesterization of its cyclohexyloxycarbonyloxy moiety

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during gastrointestinal absorption (Kondo et al., 1996). The inhibitory activity of TCV-116 itself on the specific binding of [^{125}I]angiotensin II-(Sar^1,IIe^8) to angiotensin AT_1 receptors in the rabbit aortic membrane preparations was approximately 1/250 of that of CV-11974 (Noda et al., 1993). CV-11974 was shown to be a highly potent and angiotensin AT_1 receptor-selective competitive antagonist in an angiotensin II receptor binding assay. Namely, in saturation binding experiments using rabbit aortic membranes (angiotensin AT_1 receptor), CV-11974 increased the dissociation constant (K_d) of [^{125}I]angiotensin II-(Sar^1,IIe^8) without affecting the total number of binding sites (B_{max}), whereas its binding to AT_2 receptors in bovine cerebellum membranes was negligible (Noda et al., 1993). Although CV-11974 also selectively inhibited the angiotensin II-induced contraction in isolated rabbit aorta, it reduced the maximal contractile response to angiotensin II, indicating apparently non-competitive antagonism (Noda et al., 1993; Shibouta et al., 1993). This non-competitive antagonism (or insurmountable antagonism) may be evoked by several mechanisms involved in receptor-antagonist interactions, such as (1) forming a covalent bond with receptors (irreversible antagonism), (2) dissociating slowly from receptors (pseudo-irreversible antagonism), or (3) allosteric modulation of angiotensin II receptors (Kenakin, 1987).

The purpose of the present study was to define the mechanisms of the insurmountable antagonism of CV-11974 for angiotensin II-induced rabbit aortic contraction. First, to determine whether the inhibitory effect of CV-11974 on the angiotensin II-induced maximal contractile response in the rabbit aorta was due to irreversible inhibition, we examined the effect of washing the aorta or co-incubation with losartan, a surmountable angiotensin AT_1 antagonist, on the inhibition of angiotensin II-induced contraction caused by CV-11974. Next, we focused on the second mechanism (slow dissociation from angiotensin II receptors) as described above and examined the association and dissociation kinetics of the binding of [^{3}H]CV-11974, a radioligand of CV-11974, in bovine adrenal cortical membranes, a rich source of angiotensin AT_1 receptors.

2. Materials and methods

2.1. Materials

Angiotensin I, II, and III were purchased from Peptide Institutes (Osaka, Japan). Dithiothreitol, a disulfide-reducing agent and phenylmethylsulfonyl fluoride, a protease inhibitor, were purchased from Wako (Osaka, Japan). CV-11974, losartan (Duncia et al., 1992), and PD123319 (Dudley et al., 1990) were synthesized at Takeda Chemical Industries (Osaka, Japan). Bovine serum albumin (PENTEX: fraction V fatty acid-free) was purchased from Miles (Kankakee, IL, USA). [^{125}I]Angiotensin II (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). [^{3}H]CV-11974 (21 Ci/mmol) was synthesized at Amersham Life Science (Amsersham, UK). CV-11974, losartan, and PD123319 were dissolved in dimethylsulfoxide at 1 mM or 10 mM and diluted to the desired concentration with saline for the vascular contraction study and with assay buffer for the binding study.

2.2. Contraction studies in the rabbit aorta

2.2.1. Preparation of isolated rabbit aorta

Japanese White male adult rabbits were killed by bleeding from carotid artery under sodium pentobarbital anesthesia (30 mg/kg, i.v.). The thoracic aorta was removed and cut into helical strips (2 mm in width; 2 cm in length). The strips were mounted at a resting tension of 2 g in organ baths containing 20 ml Krebs-Henseleit solution [(mM): NaCl (120), KCl (4.7), $MgSO_4 \cdot 7H_2O$ (1.2), KH_2PO_4 (1.2), $CaCl_2 \cdot 2H_2O$ (2.5), $NaHCO_3$ (25), D-glucose(10)]. The Krebs-Henseleit solution was kept at 37°C and aerated with 95% O_2 and 5% CO_2. Contractile tension was recorded on a polygraph (Recti-Horiz-8K, San-ei, Japan or Unicorder, Nihon Denshi Kagaku, Japan) using a force displacement transducer (UL-10GR, Minebea, Japan) via a strain amplifier (6 M 52, San-ei, Japan). The tissues were allowed to equilibrate for approximately 3 h before testing. During this period, they were stimulated once by the addition of angiotensin II (10 nM). After the maximal contractile response was reached (approximately 10 min later), the tissues were washed 3 times and allowed to relax to the baseline tension.

2.2.2. Interaction between CV-11974 and losartan

After equilibration, a control cumulative concentration-contractile response curve for angiotensin II was obtained. Ninety minutes after washing, the tissue was incubated first with CV-11974 (0.1 nM) or vehicle (20 μl of the diluent) for 30 min, followed by losartan (1 μM) or vehicle for another 30 min. The concentration-response curve for angiotensin II was then determined again in the presence of test compounds. In the second series of experiments, the same protocol was repeated, except that the tissue was incubated first with losartan or vehicle and then with CV-11974 or vehicle. The contractile responses are expressed as percentages of the maximal response to angiotensin II obtained from the first control concentration-response curve.

2.2.3. Recovery of the contractile response to angiotensin II after washing

After equilibration, the tissues were challenged with a single dose of angiotensin II (10 nM). The maximal contractile tension from this challenge was taken as the con-

trol response. Ninety minutes after washing, the tissue was incubated with various concentrations of CV-11974 (0.1–1 nM), losartan (10–100 nM) or vehicle for 30 min, and the contractile response to angiotensin II was first obtained in the presence of test compounds. The tissues were then washed 3 times, and this washing procedure was repeated every 30 min for 2 h. Thereafter, the tissue was rechallenged with angiotensin II, and the contractile response was observed for 30 min. To test the reversible nature of the inhibition caused by CV-11974, after washing, the contractile response to angiotensin II (10 nM) was observed over 4 h in the continuous presence of angiotensin II.

2.3. Radioligand binding studies

2.3.1. Preparation of bovine adrenal cortical membranes

The membranes were prepared according to the method of Douglas et al. (1978) with slight modification. A freshly isolated bovine adrenal cortex was homogenized 4 times with a Polytron (Brinkmann, Switzerland) (maximum speed for 1 min) in approximately 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM ethylendiaminetetraacetic acid disodium salt and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $10\,000 \times g$ at 4°C for 20 min, and the resulting supernatant was centrifuged at $20\,000 \times g$ at 4°C for 30 min. The resulting pellet (microsomal fraction) was resuspended in 50 mM Tris-HCl buffer (pH 7.4), and the suspension was stored frozen at −80°C until use. The amount of protein in the membranes was determined by the dye staining technique of Bradford (1976).

2.3.2. Angiotensin II receptor binding assays

The experiments were carried out according to the method of Chang et al. (1992) with slight modification. The binding of [^{3}H]CV-11974 or [^{125}I]angiotensin II to membranes (16 μg protein/assay tube) was examined in a final volume of 0.2 ml of 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 5 mM EDTA-2NA, 1 mM phenylmethylsulfonyl fluoride, and 0.25% bovine serum albumin with test compounds at 37°C. The binding reaction was terminated by adding 3 ml of cold saline. The solution was rapidly filtered through glass filters (Whatmann GF/B or GF/C) presoaked with saline for [^{3}H]CV-11974 binding or with assay buffer (containing 0.25% bovine serum albumin) for [^{125}I]angiotensin II binding. The filters were washed 4 times with 3 ml of ice-cold saline. The membrane-bound radioactivity of [^{3}H]CV-11974 on the filter was determined in 4 ml of a scintillation cocktail consisting of 12 l toluene, 12 g *p*-bis-(*O*-methylstyryl) benzene, 180 g 2.5-diphenyloxazole, and 5.16 l nonion (Wako) using a liquid scintillation counter (Aloka, LSC-3500), and the radioactivity of [^{125}I]angiotensin II was determined using a gamma-counter (Aloka, ARC-1000). Non-specific binding of [^{3}H]CV-11974 or [^{125}I]angiotensin II to the membrane preparation was estimated in the presence of 10 μM unlabeled CV-11974 or angiotensin II, respectively. Specific binding was defined as total binding minus non-specific binding. In saturation experiments, membranes (16 μg protein/assay tube) were incubated with various concentrations (final concentrations, 0.5–50 nM) of [^{3}H]CV-11974 for 90 min. To determine the effects of pretreatment with CV-11974 on the saturation binding of [^{125}I]angiotensin II, membranes were incubated with CV-11974 (30 nM) or assay buffer (as control) for 60 min before the addition of various concentrations (final concentrations, 0.5–5 nM) of [^{125}I]angiotensin II and another 60-min incubation. In experiments to determine ligand specificity, membranes and [^{3}H]CV-11974 (5 nM) or [^{125}I]angiotensin II (0.2 nM) were incubated with test compounds for 90 min or 60 min, respectively.

2.3.3. Kinetic analysis of [^{3}H]CV-11974 and [^{125}I]angiotensin II binding

In association experiments, the amount of specific binding of [^{3}H]CV-11974 (5 nM) and [^{125}I]angiotensin II (0.2 nM) to the membranes was determined after various time intervals between 2 and 90 min and 1 and 60 min, respectively. In dissociation experiments, [^{3}H]CV-11974 (5 nM) and [^{125}I]angiotensin II (0.2 nM) were incubated to steady state with membranes for 90 min and 60 min, respectively. The excess unlabeled ligands were then added, and the amount of specific binding after various time intervals was determined.

2.4. Data analysis

An SP123 program by H. Ono (University of Tokyo, Japan) was used for Scatchard 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 equation: $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of the radioligand and K_d is the dissociation constant obtained from Scatchard analysis and kinetic data. The IC_{50} values were determined by regression analysis, using Fieller's theorem (Finney, 1964) for the contraction studies and RS/1 (BBN Software Products, Cambridge, MA, USA) for the binding studies. The dissociation rate constant (k_{-1}) was determined from the first-order plot of In (B_t/B_{eq}) versus time, where B_{eq} and B_t are the amount of specific binding at equilibrium and time t (t: the time after the addition of unlabeled ligand). The half-life of the ligand-receptor complex ($t_{1/2}$) was calculated using $t_{1/2} = 0.693/k_{-1}$. The observed rate constant (k_{obs}) was determined from the pseudo-first-order plot of In $[B_{eq}/(B_{eq} - B_t)]$ versus time. The association rate constant (k_1) was calculated from $k_1 = (k_{obs} - k_{-1})/[L]$. K_d values from kinetic data were given as the ratio k_{-1}/k_1.

3. Results

3.1. Contraction studies in the rabbit aorta

3.1.1. Effects of losartan on CV-11974-induced antagonism of the contractile response to angiotensin II

Preincubation of aorta with CV-11974 (0.1 nM) for 30 min (vehicle + CV-11974) and 60 min (CV-11974 + vehicle) reduced the maximal response to angiotensin II by approximately 50% and 70%, respectively (Fig. 1a). In the aorta that was incubated with CV-11974 (0.1 nM) for 30 min followed by losartan (1 μM) for another 30 min, the subsequent angiotensin II concentration-response curves were shifted to the right, and the reduction of the maximal response caused by CV-11974 was almost reversed (Fig. 1a). Incubation of aorta with losartan (1 μM) before CV-11974 treatment also shifted the concentration-response curves for angiotensin II to the right, similarly to that in the presence of losartan alone (1 μM) (Fig. 1b). These results indicate that the insurmountable antagonism of CV-11974 can be reversed by the addition of a high concentration of losartan, an angiotensin AT_1-selective surmountable receptor antagonist.

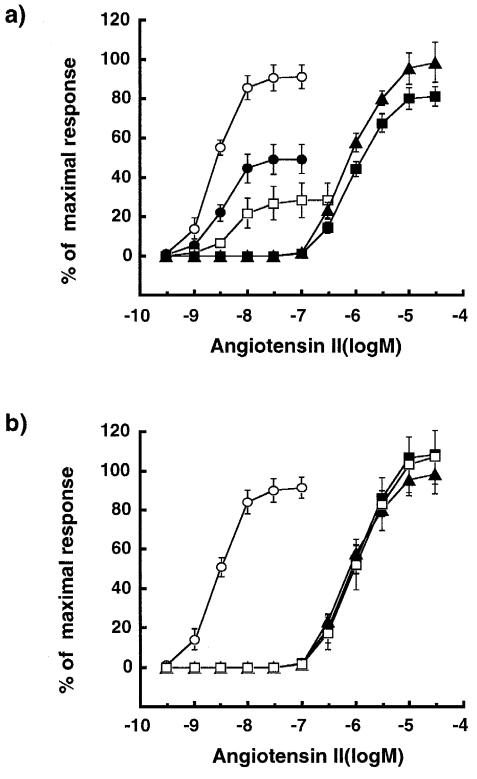

Fig. 1. Effects of co-incubation with a large amount of losartan on the inhibitory effect of CV-11974 on the concentration-contractile response curves for angiotensin II in the rabbit aorta. (a) The aorta was incubated first with CV-11974 (0.1 nM), or vehicle for 30 min and then with losartan (1 μM), CV-11974 (0.1 nM), or vehicle for an additional 30 min. (○) Vehicle + vehicle, (●) vehicle + CV-11974, (□) CV-11974 + vehicle, (■) CV-11974 + losartan, (▲) vehicle + losartan. (b) The aorta was incubated first with losartan (1 μM), or vehicle for 30 min, and then with CV-11974 (0.1 nM), losartan (1 μM), or vehicle for an additional 30 min. (○) Vehicle + vehicle, (▲) vehicle + losartan, (■) losartan + vehicle, (□) losartan + CV-11974. Data are means ± S.E.M. (n = 3–4).

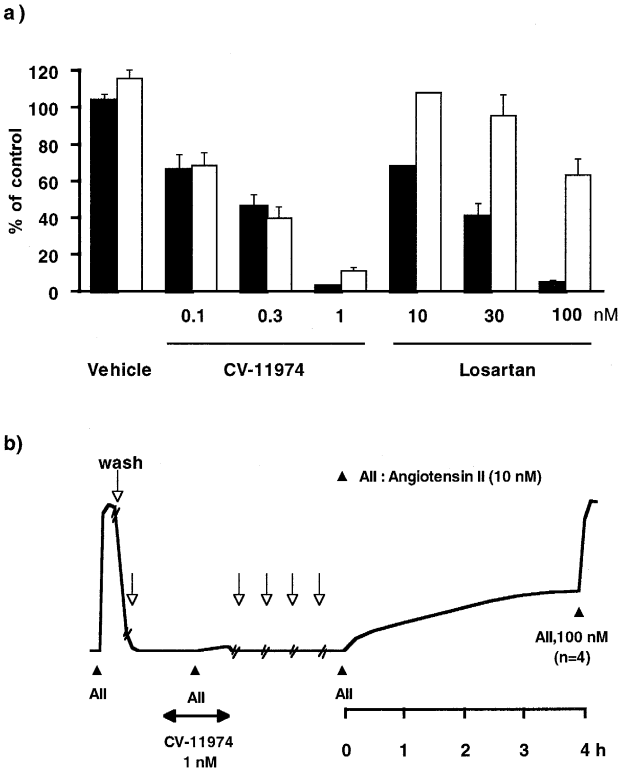

Fig. 2. Recovery of angiotensin II-induced contractions after washing of the aorta. (a) The aorta was incubated with vehicle, CV-11974 at 0.1, 0.3, or 1 nM (n = 5–10 for each), or losartan at 10 (n = 2), 30 or 100 nM (n = 5 for each) for 30 min. After the contractile response to angiotensin II (10 nM) was determined in the presence of a test compound (solid column), the aorta was washed every 30 min for 120 min. The aorta was then rechallenged with angiotensin II (10 nM), and the maximal contractile response within 30 min was measured (open column). Data are the means ± S.E.M. (b) The recovery pattern of the contractile response to angiotensin II was observed over a 4-h period after washing out CV-11974 (1 nM). Data are the means of 5 experiments. The contractile response to angiotensin II (10 nM) before drug treatment is used as a control response.

3.1.2. Effects of washing on the inhibition of the contractile response to angiotensin II by CV-11974 and losartan

Incubation of the aorta (for 30 min) with CV-11974 at 0.1–1 nM and losartan at 10–100 nM inhibited the contractile response to angiotensin II (10 nM) in a concentration-dependent manner with IC_{50} values of 0.21 nM (0.15–0.27 nM, 95% confidence limits, n = 23) and 20.3 nM (13.4–26.9 nM, 95% confidence limits, n = 12), respectively (Fig. 2a). The inhibitory effects of CV-11974 were still observed even after the aorta was washed every 30 min for 2 h (Fig. 2a). In contrast, the inhibitory effects of losartan were greatly attenuated after washing of the aorta. Even though inhibition by losartan at 100 nM was almost complete, the angiotensin II-induced contraction was restored to 63 ± 9% of the control after washing of the

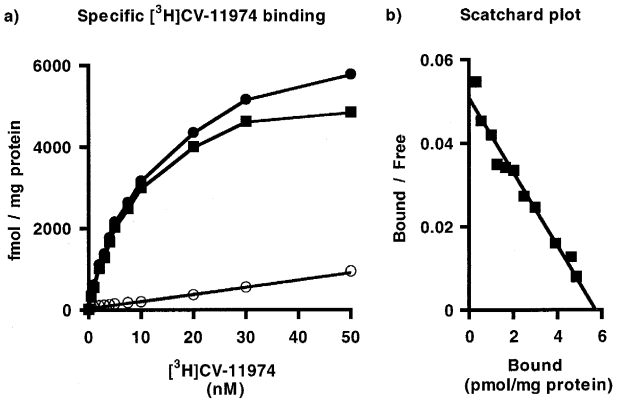

Fig. 3. (a) Saturation curve for [^{3}H]CV-11974 binding to bovine adrenal cortical membranes. Membranes were incubated with various concentrations of [^{3}H]CV-11974 for 90 min. (●) Total binding, (■) specific binding, (□) non-specific binding. (b) Scatchard plot of the same data. Points are representative of three separate experiments performed in duplicate.

aorta ($n = 5$) (Fig. 2a). When the aorta was pretreated with vehicle or losartan (100 nM), the contractile response to angiotensin II after washing reached a peak within 15 or 20 min, respectively. In contrast, the contractile response to angiotensin II after washing did not reach a peak within 30 min in the aorta pretreated with CV-11974. Therefore, in the case of CV-11974, the contractile response to angiotensin II was observed continuously for 4 h after washing. Even though the angiotensin II (10 nM)-induced contraction was almost completely inhibited by CV-11974 at 1 nM, the contraction was gradually restored to $41 \pm 7\%$ over 4 h after washing of the aorta ($n = 5$) (Fig. 2b). The additional cumulative application of angiotensin II at the end of the 4-h incubation induced a contractile response at least as great as the initial control response induced by angiotensin II (10 nM; a submaximal concentration) (Fig. 2b). These results suggest that the removal of CV-11974 from binding sites in rabbit aorta is much slower than that of losartan.

3.2. Radioligand binding studies

3.2.1. Effect of CV-11974 on the saturation binding of [^{125}I]angiotensin II in bovine adrenal cortical membranes

As previously reported (Shibouta et al., 1993), a single population of binding sites for [^{125}I]angiotensin II (0.5–5 nM) in bovine adrenal cortical membranes was found with a K_d value of 3.7 ± 0.2 nM and a B_{max} of 2.1 ± 0.2 pmol/mg protein ($n = 3$). When the membranes were preincubated with CV-11974 (30 nM) for 60 min before the addition of [^{125}I]angiotensin II, the apparent K_d increased to 7.9 (7.4, 8.3) nM, and the B_{max} of [^{125}I]angiotensin II binding decreased to 1.2 (0.8, 1.5) pmol/mg protein ($n = 2$). The Hill coefficient of [^{125}I]angiotensin II binding with and without CV-11974 was 1.00 ± 0.004 ($n = 3$) and 1.00 (1.00, 0.99), respectively.

3.2.2. Saturation analysis of [^{3}H]CV-11974

The binding of [^{3}H]CV-11974 (0.5–50 nM) to bovine adrenal cortical membranes was saturable (Fig. 3a). A Scatchard analysis of saturation binding data yielded a linear plot, suggesting the presence of a single population of binding sites. The K_d value for specific [^{3}H]CV-11974 binding was 7.4 ± 1.1 nM and the B_{max} was 5.4 ± 0.3 pmol/mg protein ($n = 3$) (Fig. 3b). A Hill plot of the [^{3}H]CV-11974 binding data revealed a Hill coefficient of 1.01 ± 0.01 ($n = 3$), indicating the absence of a positive or negative cooperative interaction.

3.2.3. Ligand specificity of [^{3}H]CV-11974 and [^{125}I]angiotensin II

Fig. 4a,b show the inhibitory effects of various angiotensin II receptor agonists and antagonists on the specific binding of [^{3}H]CV-11974 and [^{125}I]angiotensin II to bovine adrenal cortical membranes, respectively. Angiotensin I, II, III, and the angiotensin AT_1-selective receptor antagonists, losartan and CV-11974, inhibited the binding of both radioligands in a concentration-dependent,

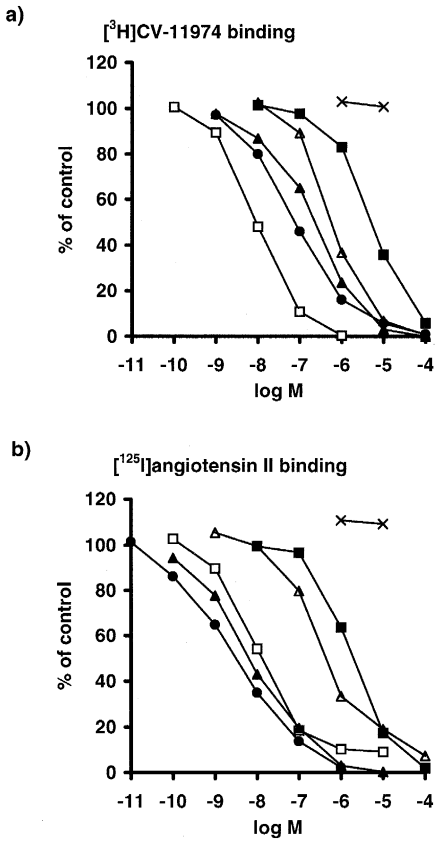

Fig. 4. Inhibition of the specific binding of (a) [^{3}H]CV-11974 and (b) [^{125}I]angiotensin II to bovine adrenal cortical membranes by various angiotensin II receptor agonists and antagonists. The membranes were incubated with (a) [^{3}H]CV-11974 (5 nM) or (b) [^{125}I]angiotensin II (0.2 nM) in the presence of CV-11974 (□), angiotensin I (■), angiotensin II (●), angiotensin III (▲), losartan (△), or PD123319 (×) for 90 or 60 min, respectively. The results are summarized in Table 1. Data are the means of 3–7 experiments performed in duplicate.

Table 1
Inhibitory effects of various angiotensin II receptor agonists and antagonists on the specific binding of [^{3}H]CV-11974 (5 nM) and [^{125}I]angiotensin II (0.2 nM) to adrenal cortical membranes

Drug	K_i (nM)	
	[^{3}H]CV-11974	[^{125}I]angiotensin II
CV-11974	4.7 ± 0.5	13 ± 1
Angiotensin II	41 ± 7	3.1 ± 1.0
Angiotensin III	100 ± 17	8.1 ± 2.6
Angiotensin I	2609 ± 206	1794 ± 439
Losartan	303 ± 22	431 ± 6
PD-123319	≫ 1000	≫ 1000

K_i values were calculated according to the formula $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the radioligand concentration and K_d is the dissociation constant of the radioligand as determined by saturation and kinetic studies. Values are the means ± S.E.M. of 3–7 experiments performed in duplicate.

monophasic manner. In contrast, PD123319, a selective angiotensin AT_2 receptor antagonist, had no effect on either, even at the high concentration of 10 μM. Table 1 summarizes the inhibitory constants (K_i) for these drugs. The specific binding of [^{3}H]CV-11974 and [^{125}I]angiotensin II was inhibited most potently by each unlabeled ligand, CV-11974 and angiotensin II, respectively. However, the rank order of potencies of angiotensin I, II, III, and losartan for the inhibition of [^{3}H]CV-11974 binding correlated with those for [^{125}I]angiotensin II (angiotensin II > angiotensin III > losartan > angiotensin I).

Recent analyses of receptors with site-directed point mutations have revealed that two disulfide bridges formed by pairs of Cys residues in the extracellular domain may be important for angiotensin II binding but less important for [^{3}H]losartan binding (Yamano et al., 1992). Therefore, the effect of dithiothreitol, a disulfide-reducing agent, on both types of bindings was examined. Dithiothreitol inhibited [^{125}II]angiotensin II binding in a dose-dependent manner with an IC_{50} value of 5.8 ± 0.3 mM ($n = 3$). Although the effects of dithiothreitol could not simply be compared because the concentration of [^{3}H]CV-11974 was 25 times higher than that of [^{125}I]angiotensin II, [^{3}H]CV-11974

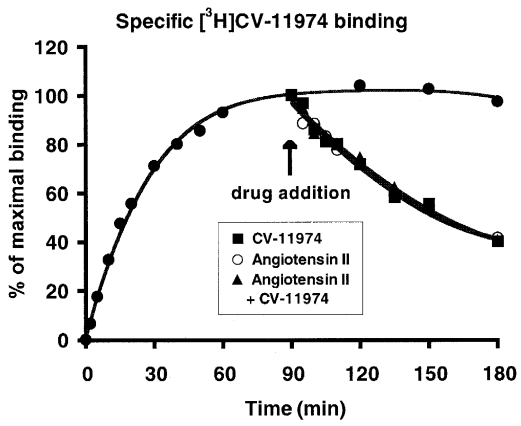

Fig. 5. Time-course of the association and dissociation of [^{3}H]CV-11974 binding to bovine adrenal cortical membranes. After [^{3}H]CV-11974 (5 nM) binding to membranes had reached equilibrium (at 90 min), dissociation was induced by the addition of unlabeled CV-11974 (10 μM; ■), angiotensin II (100 μM; ○), or a combination (▲) of CV-11974 (10 μM) and angiotensin II (100 μM). The specific binding is expressed as a percentage of the specific binding at equilibrium (maximal binding). Data are the means of 2–3 experiments performed in duplicate.

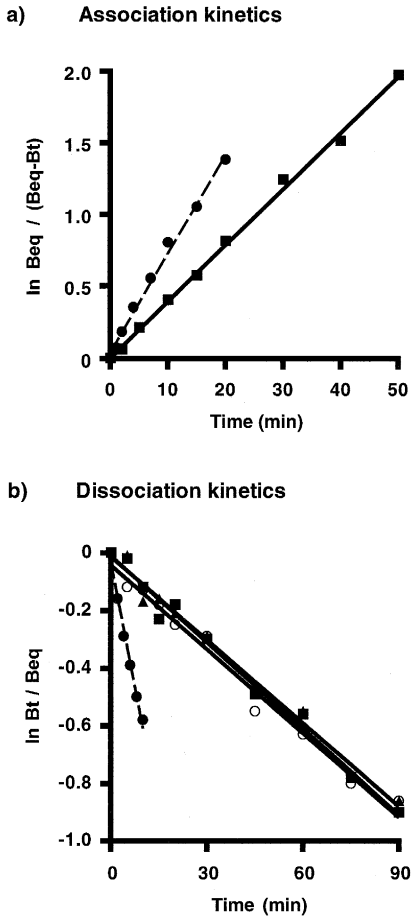

Fig. 6. Association and dissociation kinetics of the specific binding of [^{3}H]CV-11974 and [^{125}I]angiotensin II to bovine adrenal cortical membranes. (a) Pseudo-first-order kinetic plots of initial binding of [^{3}H]CV-11974 (5 nM; ■) and [^{125}I]angiotensin II (0.2 nM; ●) to membranes. On the ordinate, B_{eq} is the amount of specific binding at equilibrium, and B_t is the amount of specific binding at time t. The slope of the plot is the observed rate constant (k_{obs}) for the pseudo-first-order reaction. The second-order association rate (k_1) was calculated as described in Materials and methods. (b) Dissociation kinetics of specific binding of [^{3}H]CV-11974 (5 nM; solid line) and [^{125}I]angiotensin II (0.2 nM; broken line) to membranes. After [^{3}H]CV-11974 or [^{125}I]angiotensin II binding to membranes reached equilibrium, dissociation of [^{3}H]CV-11974 or [^{125}I]angiotensin II was induced by the addition of unlabeled CV-11974 (10 μM; ■), angiotensin II (100 μM; ○), or a combination (▲) of CV-11974 (10 μM) and angiotensin II (100 μM) or the addition of unlabeled angiotensin II (10 μM; ●), respectively. On the ordinate, B_{eq} and B_t are binding at equilibrium and time t (t: time after addition of the unlabeled ligand). The slope of the first-order plot is the dissociation rate constant (k_{-1}). Data are representative of 2–3 separate experiments performed in duplicate.

binding was moderately affected by dithiothreitol (approximate IC_{50} = 123 mM, $n = 3$).

3.2.4. Kinetics of [^{3}H]CV-11974 and [^{125}I]angiotensin II binding

Fig. 5 shows the time-course of association and dissociation of [^{3}H]CV-11974 (5 nM) binding to bovine adrenal cortical membranes. The specific binding of [^{3}H]CV-11974 increased slowly with time and reached equilibrium after approximately 90 min. This equilibrium was maintained for up to 180 min of incubation. The specific binding of [^{125}I]angiotensin II (0.2 nM) increased more rapidly than that of [^{3}H]CV-11974 and reached equilibrium after approximately 60 min. The association rate constant (k_1) of [^{3}H]CV-11974 and [^{125}I]angiotensin II was calculated from these studies to be 0.0059 (0.0059, 0.0059) and 0.0660 (0.0670, 0.0650) min^{-1} nM^{-1}, respectively (Fig. 6a) (n = 2).

After the specific binding of [^{3}H]CV-11974 (5 nM) and [^{125}I]angiotensin II (0.2 nM) reached equilibrium, dissociation was induced by the addition of 10 μM unlabeled CV-11974 and angiotensin II, respectively (Fig. 6b). CV-11974 caused the slow release of radioactivity from the [^{3}H]CV-11974-receptor complex with a half-time of 66 ± 3 min ($n = 3$). The dissociation rate constant (k_{-1}) of [^{3}H]CV-11974 was calculated to be 0.0104 ± 0.0004 min^{-1} ($n = 3$). The dissociation of [^{3}H]CV-11974, caused by the addition of angiotensin II (100 μM) both with and without CV-11974 (10 μM), was identical to that caused by the addition of CV-11974 alone (dissociation rate constants were: upon addition of angiotensin II, K_{-1} = 0.0097 ± 0.0001 min^{-1}; angiotensin II + CV-11974, k_{-1} = 0.0100 (0.0103, 0.0096) min^{-1}) (Fig. 6b) (n = 2–3). In contrast, the dissociation of [^{125}I]angiotensin II caused by the addition of angiotensin II (10 μM) was rapid with an initial half-time of 12 ± 1 min (Fig. 6b) ($n = 3$). The initial dissociation rate constant (k_{-1}) of [^{125}I]angiotensin II was calculated to be 0.0571 ± 0.0036 min^{-1} ($n = 3$), indicating that the dissociation rate of [^{125}I]angiotensin II from bovine adrenal cortical membranes was 5 times faster than that of [^{3}H]CV-11974.

4. Discussion

We examined the properties of the angiotensin II antagonism caused by CV-11974 in contraction studies with rabbit aorta, and the kinetics of [^{3}H]CV-11974 binding using bovine adrenal cortical membranes. These preparations were chosen because they express mainly the angiotensin AT_1 receptor (Wong et al., 1990; Chang and Lotti, 1991; Noda et al., 1993; Shibouta et al., 1993; Sasaki et al., 1991). Although the rabbit aortic angiotensin AT_1 receptor has not yet been cloned, several highly similar cDNAs encoding AT_1 receptors have been cloned from bovine adrenal gland, rat vascular smooth muscle, rat kidney, bovine kidney, rat liver, and human liver (Sasaki et al., 1991; Murphy et al., 1991; Iwai et al., 1991; Yoshida et al., 1992; Takayanagi et al., 1992). These angiotensin AT_1 receptors, including rabbit aorta and bovine adrenal gland receptors, are considered to have similar pharmacological properties such as ligand binding characteristics in the competition binding experiment using angiotensin antagonists and agonists and calcium mobilization (Sasaki et al., 1991; Murphy et al., 1991). Furthermore, Aiyar et al. (1995) showed that in kinetic studies using bovine adrenal cortical membranes, the dissociation rate of SB 203220, which exhibits a partial insurmountable antagonism of angiotensin II-induced contraction in rabbit aorta, is slower than that of SK&F 108566, a surmountable antagonist. Thus, we used the bovine adrenal cortical membranes in the binding studies.

We have reported previously that CV-11974 reduces the maximal contractile response to angiotensin II in a concentration-dependent manner in rabbit aorta, indicating insurmountable antagonism (Shibouta et al., 1993). However, in the present studies, we observed that the reduction in the angiotensin II-induced maximal response caused by CV-11974 (0.1 nM) was reversed almost completely by the addition of a high concentration of losartan (1 μM), a surmountable angiotensin AT_1 receptor antagonist (Fig. 1). These results suggest that most of the CV-11974 bound to the receptors may be replaced by losartan, and then subsequently added angiotensin II may compete for the receptors occupied by losartan. Thus, CV-11974 and losartan may interact with the same binding sites, and it is unlikely that CV-11974 binds irreversibly to angiotensin II receptors.

One explanation for the insurmountable antagonism exhibited by angiotensin AT_1 receptor antagonists is their pseudo-irreversible antagonism, which is thought to be produced by slow dissociation of the antagonist-receptor complex (Robertson et al., 1992; Wienen et al., 1992; Panek et al., 1995). To test this hypothesis, we performed washout experiments on angiotensin II-induced contraction using rabbit aorta, and receptor binding experiments using [^{125}I]angiotensin II in bovine adrenal cortical membranes.

Washout experiments: the inhibitory effect of CV-11974 on angiotensin II-induced contractions was still observed after washing of the aorta. In contrast, the inhibitory effect of losartan was greatly attenuated after washing of the aorta (Fig. 2a). However, the inhibition of angiotensin II-induced contractions by CV-11974 was not irreversible: even though the angiotensin II-induced contractions were almost completely inhibited by CV-11974, the contractile response to angiotensin II was gradually restored over 4 h after washing of the aorta (Fig. 2b). These results suggest that CV-11974 is a reversible and possibly slowly dissociating receptor antagonist. However, additional factors that may influence washout of the antagonists in the whole tissues, including lipid solubility or receptor environment, may contribute to the insurmountable antagonism by CV-

11974 as explained for other angiotensin II receptor antagonists (Panek et al., 1995). Therefore, an alternative explanation is that the persistent angiotensin II antagonism seen with CV-11974 may be due partly to slow removal of CV-11974 from compartments within tissues, cells, or the surrounding matrix.

Binding experiments: previous saturation binding studies have indicated that CV-11974 inhibits the binding of angiotensin II in bovine adrenal cortical and rabbit aortic membrane fractions in a competitive manner (Noda et al., 1993; Shibouta et al., 1993). However, it should be noted that CV-11974 and angiotensin II were added simultaneously in those binding studies, whereas in the contraction studies the rabbit aorta was preincubated with CV-11974 before the addition of angiotensin II. Therefore, in the present studies, we examined the effects of preincubation with CV-11974 on [^{125}I]angiotensin II binding. In contrast to previous reports, preincubation of membranes with CV-11974 for 60 min increased the apparent $K_{d,}$ and decreased the B_{max} of [^{125}I]angiotensin II binding, indicating apparently non-competitive antagonism. This insurmountable behavior of CV-11974 could be explained as follows: the interaction of CV-11974-receptor complexes with subsequently added angiotensin II may not reach equilibrium within the 60-min incubation period due to the slow dissociation of CV-11974 from its binding sites (pseudo-irreversible antagonism). Similar findings have been reported for [^{125}I]angiotensin II binding experiments using angiotensin AT_1 receptors of rat liver membranes (Hara et al., 1995). To verify this mechanism, we examined the binding properties of [^{3}H]CV-11974.

The binding of [^{3}H]CV-11974 to bovine adrenal cortical membranes was slow, time-dependent, reversible, and saturable. Scatchard and Hill plot analyses of [^{3}H]CV-11974 binding indicated the presence of a single population of binding sites with a K_d 7.4 nM and a B_{max} of 5.4 pmol/mg protein and the absence of negative or positive cooperation. [^{3}H]CV-11974 binding was completely inhibited by various angiotensin peptides and losartan, whereas it was not affected by PD123319, a specific angiotensin AT_2 receptor antagonist (Fig. 4). Furthermore, the rank order of inhibitory potency of angiotensin I, II, III, and losartan for [^{3}H]CV-11974 binding correlated with that for [^{125}I]angiotensin II binding. Therefore, it is suggested that the majority of [^{3}H]CV-11974 binding sites in bovine adrenal cortical membranes are angiotensin AT_1 receptors. However, the relative inhibitory potencies of CV-11974 and angiotensin II for each radioligand were slightly different, since the binding was inhibited most potently by the respective unlabeled ligand (Table 1). The reason for this difference is not clear, but it may be that the binding sites for angiotensin II and CV-11974 on the angiotensin AT_1 receptor do not overlap totally, as recent reports have indicated that the essential sites involved in binding epitopes for angiotensin II are not identical to those for non-peptide antagonists, using mutated angiotensin II receptors (Hjorth et al., 1994; Ji et al., 1994; Yamano et al., 1995).

In our kinetic studies, [^{125}I]angiotensin II dissociated rapidly with an initial half-time of dissociation ($t_{1/2}$) of 12 min in adrenal cortical membranes, which is consistent with results of a previous study using the membrane fraction of bovine adrenal cortex ($t_{1/2}$ of [^{125}I]angiotensin II = 13–23 min) (Glossman et al., 1974). It has been shown that [^{3}H]DuP 753, a radioligand of losartan, dissociated rapidly from angiotensin II binding sites in rat adrenal microsomes ($t_{1/2}$ of [^{3}H]DuP 753 = 2.5 min) (Chiu et al., 1990). On the other hand, the dissociation rate of [^{3}H]CV-11974 was 5 times slower than that of [^{125}I]angiotensin II ($t_{1/2}$ of [^{3}H]CV-11974 = 66 min). Furthermore, [^{125}I]EXP985, a close structural analog of losartan which, like CV-11974, showed insurmountable antagonism in vascular contraction studies, also dissociates slowly from angiotensin II binding sites ($t_{1/2}$ of [^{125}I]EXP985 = 58 min) (Chiu et al., 1992). These findings suggest that the insurmountable antagonism caused by CV-11974 is attributable to its slow dissociation from angiotensin AT_1 receptors.

Another possible explanation for this insurmountable antagonism is the allosteric modulation of angiotensin II receptors (allosteric antagonism), which reduced the binding capacity of the receptor for the agonist (as has been postulated, at least in part, for EXP3174: Wienen et al., 1992). However, the rate of dissociation of [^{3}H]CV-11974 from angiotensin AT_1 receptors after the addition of excess unlabeled CV-11974 did not differ from that following the addition of excess angiotensin II, both with and without CV-11974 in bovine adrenal cortical membranes. Furthermore, the rate of dissociation of [^{125}I]angiotensin II-(Sar^1,IIe^8) from angiotensin AT_1 receptors resulting from the addition of excess CV-11974 also did not differ from that caused by the addition of excess angiotensin II in rabbit aortic membranes (Noda et al., 1993). It is unlikely, therefore, that CV-11974 binds to allosteric sites.

It has been suggested that the carboxyl group in the 7-position on the benzimidazole of CV-11974 or in the 5-position on the imidazole ring of EXP 3174 is important for the observed insurmountable antagonism (Noda et al., 1993; Wienen et al., 1992), although the actual molecular interaction with angiotensin AT_1 receptors has not yet been clarified. Therefore, the carboxyl group might contribute to the slow dissociation rate of CV-11974. However, the exact role of the carboxyl group remains unclear, and further studies are required.

The inhibitory effect of CV-11974 was more potent in the contraction studies than in receptor binding studies. This discrepancy may be attributable to the differences in experimental conditions (e.g., the presence or absence of bovine serum albumin, whole tissues, or membranes). It has been shown that bovine serum albumin modulates the binding of non-peptide AT_1 receptor antagonists, especially diacidic antagonists such as DuP532 (Chiu et al.,

1991). In a preliminary experiment it was observed that when a lower concentration (0.05%), instead of the usual concentration (0.25%), of bovine serum albumin was used, the inhibitory effect of CV-11974 on [^{125}I]angiotensin II binding to the bovine adrenal cortical membranes was enhanced 14-fold. In contrast, the inhibitory effect of CV-11974 was decreased about 10-fold with 0.25% bovine serum albumin in the aortic contraction assay. Another explanation for this discrepancy could be the difference in the relative density of receptors and agonist between rabbit aortic tissues and bovine adrenal cortical membranes and differences in the receptor environment.

In conclusion, the present studies have demonstrated that the inhibitory effect of CV-11974 on angiotensin II-induced contraction in rabbit aorta was more persistent than that of losartan, but was reversible. We have demonstrated that [^{3}H]CV-11974 binds specifically to angiotensin AT_1 receptors and associates slowly with and dissociates slowly from the binding sites in bovine adrenal cortical membranes. Thus, the insurmountable antagonism of angiotensin II-induced contractions by CV-11974 in rabbit aorta may be attributable to its slow dissociation from angiotensin AT_1 receptors, and this kinetic characteristic may be related to the prolonged angiotensin II antagonistic action of TCV-116, a prodrug of CV-11974, in vivo.

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