British Journal of Pharmacology (1995) 114, 447-453

Species-related differences in inotropic effects of angiotensin II in mammalian ventricular muscle: receptors, subtypes and phosphoinositide hydrolysis

Akira Ishihata & [1]Masao Endoh

Department of Pharmacology, Yamagata University School of Medicine, Yamagata 990-23, Japan

1 Experiments were carried out to clarify the mechanisms responsible for variations in the positive inotropic effect (PIE) of angiotensin II (AII) on ventricular muscles from various mammals. We examined the density of AII receptors, the relative proportions of receptor subtypes and the acceleration of the hydrolysis of phosphoinositide that was induced by AII, as well as the PIE of AII in ventricular muscles from the rabbit, dog, rat and ferret.

2 In the rabbit, AII (1 μM) in the presence of bupranolol (0.3 μM) and prazosin (0.1 μM) elicited a concentration-dependent PIE, which was antagonized by a selective AT_1 subtype antagonist, losartan, but not by an AT_2 antagonist, PD123319. AII did not have any inotropic effects in ventricular muscles from the dog, rat and ferret.

3 Specific high-affinity binding of [^{125}I]-AII, with a similar K_d value in each case (1–2 nM), was observed with membrane fractions derived from ventricular muscle of all four species tested.

4 In the rabbit, losartan and PD123319 each displaced approximately 50% of [^{125}I]-AII specific binding having high affinity for the receptors, and indicating that AT_1 and AT_2 subtypes were present in equal numbers. In the other species the AT_1 subtype of receptors was predominant.

5 In all four species AII caused a concentration-dependent acceleration of the hydrolysis of phosphoinositide in ventricular slices that had been prelabelled with *myo*-[^{3}H]-inositol.

6 The results indicate that the signal-transduction process distal to acceleration of the hydrolysis of phosphoinositide may be responsible for the wide range of species variations in the inotropic action of AII on mammalian ventricular myocardium.

Keywords: Angiotensin II; AT_1 receptor; AT_2 receptor; positive inotropic effect; species difference; hydrolysis of phosphoinositide; binding of [^{125}I]-angiotensin II; losartan; PD123319

Introduction

Angiotensin II (AII) elicits a positive inotropic effect (PIE) in the cardiac muscles of certain mammalian species that include man (Moravec *et al.*, 1990) and the rabbit (Dempsey *et al.*, 1971; Freer *et al.*, 1976; Ishihata & Endoh, 1993). Direct activation of specific receptors by AII is considered to trigger a signal-transduction process that results in the PIE (Dempsey *et al.*, 1971; Freer *et al.*, 1976; Kobayashi *et al.*, 1978; Moravec *et al.*, 1990). However, the AII-induced PIE varies widely among different mammalian species. In addition to AII receptor density differences between species, the presence of two subtypes of AII receptors, AT_1 and AT_2, may contribute to these species-dependent variations.

AT_1 receptors consist of 359 amino acid residues with seven putative membrane-spanning domains and they belong to the family of G protein-coupled receptors (Sasaki *et al.*, 1991; Iwai *et al.*, 1991). AT_2 receptors, on the other hand, appear not to interact with G proteins (Bottari *et al.*, 1991). Nevertheless, the receptors can be distinguished using the nonpeptide AT_1-selective antagonist losartan and the AT_2-selective antagonist PD123319 (Whitebread *et al.*, 1989; Dudley *et al.*, 1990). It has been shown that both subtypes are present in rabbit ventricular myocardium (Rogg *et al.*, 1990).

Activation of AII receptors in cardiac muscle is coupled to various signal-transduction processes, which include activation of the slow Ca^{2+} channel (Freer *et al.*, 1976) and acceleration of the hydrolysis of phosphoinositide (Leung *et al.*, 1986; Baker & Singer, 1988; Baker *et al.*, 1989; Baker & Aceto, 1989). We recently demonstrated that, in the rabbit ventricular muscle, the PIE of AII is elicited via activation of AT_1 receptors in association with acceleration of the hydrolysis of phosphoinositide (Ishihata & Endoh, 1993). In the present study, the density of AII receptors, the relative proportion of AT_1 and AT_2 receptor subtypes and the acceleration of the hydrolysis of phosphoinositide induced by AII have each been determined in the ventricular muscles of the rabbit, dog, rat and ferret. The results are discussed in terms of the mechanisms responsible for the wide range of species-dependent variations in the AII-induced PIE on mammalian cardiac muscle.

Methods

Isolation of ventricular muscles and experimental procedures

Male albino rabbits weighing 1.5–2.2 kg, male Wistar rats weighing 250–350 g, mongrel dogs weighing 5–10 kg and male ferrets weighing 1.5–2.0 kg were used for the experiments. The rabbits were anaesthetized by intravenous administration of sodium pentobarbitone (35 mg kg^{-1}) into a marginal ear vein; 1000 u kg^{-1} heparin sodium was administered simultaneously. The heart was removed immediately, and two or three papillary muscles were excised from the right ventricle. Rats and ferrets were lightly anaesthetized with ether and heparinized (1500 u kg^{-1}, i.p.), and right ventricular papillary muscles were isolated. Dogs were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.) and right ventricular trabeculae were excised. Muscles were mounted in 20 ml organ baths that contained Krebs-

[1] Author for correspondence.

Henseleit solution (plus 0.057 mM ascorbic acid and 0.027 mM disodium EDTA to prevent the autoxidation of compounds examined). The solution was bubbled with 95% O_2 and 5% CO_2 at 37°C and the pH of the solution was 7.4 when bubbled with the gas mixture. Concentrations of the various constituents of the solution were as follows (mM): Na^+ 142.9, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, SO_4^{2-} 1.2, Cl^- 127.8 and glucose 11.1. In the case of rat papillary muscles, the extracellular concentration of Ca^{2+} ions was reduced to 1.25 mM. Ventricular muscles were electrically stimulated by square-wave pulses of 5 ms duration and a voltage that was approximately 20% greater than the threshold. Pulses were applied at a frequency of 1 Hz for rabbits, rats and ferrets and 0.5 Hz for dogs. The developed tension was recorded on a thermal pen-writing oscillograph (Recti-Horiz-8K; NEC-San-ei Instrument, Tokyo, Japan) via strain-gauge transducers (Shinkoh UL 10 GR; Minebea, Tokyo, Japan). During the 60 min equilibration period, papillary muscles were stretched to a length that gave 90% of the maximal contractile force. (±)-Bupranolol (0.3 μM) and prazosin (0.1 μM) were allowed to act for 20 min before the administration of AII and were both present in the solution in the organ bath throughout the experiments to avoid any interference by α- and β-adrenoceptor stimulation as a result of the release of catecholamines (Dempsey *et al.*, 1971). At the end of each experiment, the maximal contractile force was determined in each muscle by administration of isoprenaline (1–10 μM) after washing out of other drugs for at least 1 h and the basal force of contraction had returned to the control level. The concentration was elevated in a stepwise manner until the maximal response was achieved.

Radioligand-binding assay

Details of the experimental procedures used to prepare the membrane fraction and of the binding assay were essentially the same as those described previously by Takanashi & Endoh (1991). In brief, pieces of ventricular muscle, including free walls and septum (rabbits, rats or ferrets) or right ventricular free wall (dogs), were excised and homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose containing 5 mM tris(hydroxymethyl)aminomethane (Tris)-HCl and 1 mM $MgCl_2$, pH 7.4) in a Polytron PT-10 (Kinematica, Lucerne, Switzerland) with three bouts of 15 s each at a setting of 7. The homogenate was centrifuged at 500 *g* for 15 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and centrifuged at 50,000 *g* for 20 min at 4°C. The resultant pellet was washed twice with ice-cold incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) by repeated resuspension and recentrifugation. The final pellet was resuspended in ice-cold incubation buffer that contained 2 mg ml^{-1} bovine serum albumin and 0.2 mg ml^{-1} bacitracin.

[^{125}I]-angiotensin II binding to membrane fractions was carried out as follows. The incubation mixture contained 150 μl of a suspension of membranes (approximately 150–300 μg of protein), 50 μl of a solution of [^{125}I]-angiotensin II at various concentrations (specific activity, ca. 2,000 Ci $mmol^{-1}$) and 50 μl of incubation buffer (for total binding) or unlabelled AII (1 μM; for nonspecific binding). In the experiments to examine displacement of [^{125}I]-AII, the incubation mixture contained 150 μl of the membrane fraction, 50 μl of [^{125}I]-AII (0.25 nM) and 50 μl of a solution of unlabelled competitor at various concentrations. The incubation, started by the addition of the membrane fraction, was carried out for 60 min at 25°C and terminated by addition of 2 ml of ice-cold incubation buffer. Reaction mixtures were subjected to rapid filtration under reduced pressure through glass-fibre Whatman GF/C filters (presoaked in incubation buffer that contained 2 mg ml^{-1} bovine serum albumin), using a Brandel 24R cell-harvester (Brandel, Gaithersburg, MD, U.S.A.). The filters were immediately washed three times with 4 ml of ice-cold incubation buffer. After drying of filters (1 h at 90°C) the radioactivity trapped on to the filters was quantitated with an automatic gamma counter (1470 WIZARD; Wallac Oy, Turku, Finland) at an efficiency of more than 78%.

Nonspecific binding of [^{125}I]-AII was defined as the radioactivity that bound to membrane fractions that was not displaced by a high concentration of unlabelled AII (1 μM). Specific binding of [^{125}I]-AII was defined in terms of total radioactivity minus radioactivity due to nonspecific binding. Each binding assay was carried out in duplicate. Protein was quantitated by the method of Lowry *et al.* (1951).

Quantitation of [^{3}H]-inositol phosphates

Hearts were quickly removed from rabbits, rats, dogs and ferrets, and placed in Krebs-Henseleit solution that was bubbled with 95% O_2:5% CO_2 at 4°C. The experimental procedure was the same as that described previously (Ishihata & Endoh, 1993). In brief, ventricular slices (0.5 mm thick) were prepared with a tissue slicer (Thomas, Philadelphia, PA, U.S.A.) and were equilibrated in Krebs-Henseleit solution for 30 min. Each experiment was carried out in duplicate. Each tube contained one slice of ventricle that had been cut and trimmed to an appropriate size (ca. 40 mg and 0.5 mm in thickness). The slice in each tube was preincubated with 24 μCi *myo*-[^{3}H]-inositol in 4 ml Krebs-Henseleit solution for 120 min. Then the solution was changed to fresh solution that contained 5 mM *myo*-inositol and 10 mM LiCl, and all the experiments were performed in the presence of this Li^+-containing solution. (±)-Bupranolol (0.3 μM) and prazosin (0.1 μM) were added to the solution 20 min before administration of an agonist to avoid any interference by activation of adrenoceptors. At the end of the incubation, the slices were quickly blotted, frozen in liquid nitrogen and weighed. Then the slices were put into 1.0 ml of a mixture of chloroform, methanol and 12 N HCl (100:200:1, v/v). After the addition of 0.2 ml of 5 mM EDTA, the tissue was homogenized with a homogenizer (Polytron PT-10). The tip of the homogenizer was rinsed with 0.5 ml of a mixture of chloroform, methanol, 12 N HCl and 5 mM EDTA (100:200:1:80, v/v), and the rinsing fluid was added to the original solution. Chloroform (0.4 ml) and 5 mM EDTA (0.5 ml) were added sequentially and the samples were centrifuged at 1,400 *g* for 20 min to separate the aqueous and organic phases. An aliquot of the aqueous layer was removed, neutralized with 1 M KOH and applied to column that contained a 50% slurry of AG1-X8 (anion-exchange resin, 100–200 mesh, formate form; Bio-Rad, Richmond, CA, U.S.A.). Each column was washed first with 20 ml distilled water, and the resulting glycerophosphoryl esters were eluted with 8 ml 5 mM sodium tetraborate-60 mM sodium formate as described previously (Berridge *et al.*, 1983; Takanashi & Endoh, 1991). Then [^{3}H]-inositol phosphates were eluted by 0.2 M ammonium formate in 0.1 M formate as described by Berridge *et al.* (1983). Aliquots of the eluate were monitored for radioactivity, after addition to a scintillation mixture (ACS-II; Amersham, Arlington Heights, IL, U.S.A.) in a scintillation counter (Tri-Carb 1500; Packard, Downers Grove, IL, U.S.A.) with an efficiency of 66%. Since it had been determined in a preliminary experiment that the radioactivity of [^{3}H]-inositol trisphosphate (IP_3) and of [^{3}H]-inositol bisphosphate was low, probably because of their rapid metabolism to [^{3}H]inositol monophosphate (IP_1), IP_1 was used as an indicator of the hydrolysis of phosphoinositide (Ishihata & Endoh, 1993).

Drugs

The drugs used were obtained from the sources listed in parentheses: prazosin hydrochloride (Pfizer Taito, Tokyo, Japan); (±)-bupranolol HCl (Kaken Pharmaceutical, Tokyo, Japan); AII (Peptide Institute, Osaka, Japan); saralasin ([Sar^1, Val^5, Ala^8]angiotensin II; Sigma Chemical,

St. Louis, MO, U.S.A.); losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazole-5-yl)biphenyl-4-yl)methyl] imidazole, potassium salt; Du Pont, Wilmington, DE, U.S.A.); (−)-isoprenaline hydrochloride, *myo*-inositol and lithium chloride (Sigma Chemical, St. Louis, MO, U.S.A.); *myo*-[^{3}H]inositol (specific activity 115.5 Ci.mmol^{-1}; Amersham, Buckinghamshire, UK); PD123319-121b ((S)-1-[[4-dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid, ditrifluoroacetate, dihydrate; Warner-Lambert, Ann Arbor, MI, U.S.A.). The stock solution of isoprenaline was prepared in 1% w/v ascorbic acid, further diluted with 0.9% w/v NaCl solution and kept ice-cold.

Analysis of data and statistics

In the radioligand-binding assay, the maximum number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d) were determined from a Scatchard plot. Analysis of the curve for the displacement of [^{125}I]-AII by nonlinear curve-fitting was performed by use of the LIGAND programme. The inhibition constant (K_i) was calculated from the following formula: $K_i = IC_{50}(1 + L^*.K_d^{-1})^{-1}$, where L^* is the concentration of radioactive ligand used and K_d is the dissociation constant. Experimental values are presented as means ± s.e.mean. Statistical analysis was performed by one-way analysis of variance, and when the F ratio indicated a significant difference, differing pairs were compared by Tukey's test. A P value of less than 0.05 was considered to indicate a significant difference.

Results

Inotropic effects of angiotensin II on ventricular muscles from different mammalian species

Figure 1 shows the effects of AII on the force of contraction in the presence of bupranolol (0.3 μM) and prazosin (0.1 μM) on ventricular muscles isolated from the rat, dog and ferret. AII (1 μM) did not have any inotropic effect in rat and ferret papillary muscles, or in dog ventricular trabeculae. However isoprenaline, administered after washing out of the drugs for 1 h elicited a concentration-dependent PIE in these preparations. By contrast, in the rabbit ventricular papillary muscle, AII elicited a concentration-dependent PIE (Figure 2). The PIE was inhibited by the selective AT_1-receptor antagonist, losartan (at 1 and 10 μM, Figure 2a), but not by PD123319 (Figure 2b).

Characteristics of binding of [^{125}I]-angiotensin II to ventricular muscles from different mammalian species

Figure 3a shows results of a representative experiment, namely analysis of the saturation binding of [^{125}I]-AII to a membrane fraction derived from rabbit ventricular muscle, indicating that specific high-affinity binding was saturable. Analysis of the data as a Scatchard plot indicated the presence of an apparently unique class of binding sites (Figure 3b) with a mean K_d value of 0.97 nM and a B_{max} of 37.3 fmol mg^{-1} protein (Table 1). Specifically bound [^{125}I]-AII (0.25 nM) was displaced by saralasin and AII in a monophasic manner, which suggested the presence of a single class of binding sites with high affinity for [^{125}I]-AII (Figure 4). These results are in agreement with previous findings in the rabbit (Mukherjee *et al.*, 1982; Baker *et al.*, 1984; Rogg *et al.*, 1990). By contrast, losartan and PD123319 displaced specifically bound [^{125}I]-AII in a biphasic manner, an indication of the presence of two subtypes of AII receptor in the rabbit myocardium (Figure 4). In the presence of PD123319 (10 μM), losartan displaced [^{125}I]-AII from the remaining binding sites and these sites had a high affinity for losartan ($pK_i = 7.89 \pm 0.11$, $n = 3$), as shown in Figure 5a. By contrast, in the presence of 1 μM of losartan, PD123319 displaced the remaining bound [^{125}I]-AII in a monophasic manner and bound to receptors with high affinity ($pK_i = 7.13 \pm 0.17$, $n = 4$) (Figure 5b). From the results of the displacement experiments using these selective antagonists, we estimated that the AT_1 and AT_2 subtypes accounted for 46% and 54% of the total population of AII

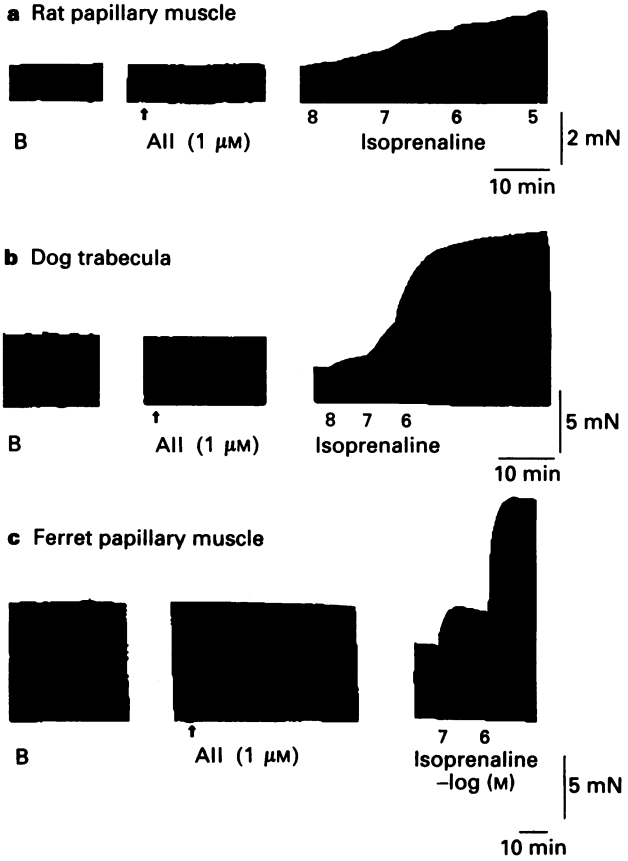

Figure 1 Influence of angiotensin II (AII, 1 μM) on the force of contraction in isolated right ventricular muscles from (a) the rat, (b) dog and (c) ferret in the presence of 0.3 μM bupranolol and 0.1 μM prazosin. The effects of isoprenaline after washing out of the drugs are shown in the right panel for comparison. B, Basal force of contraction.

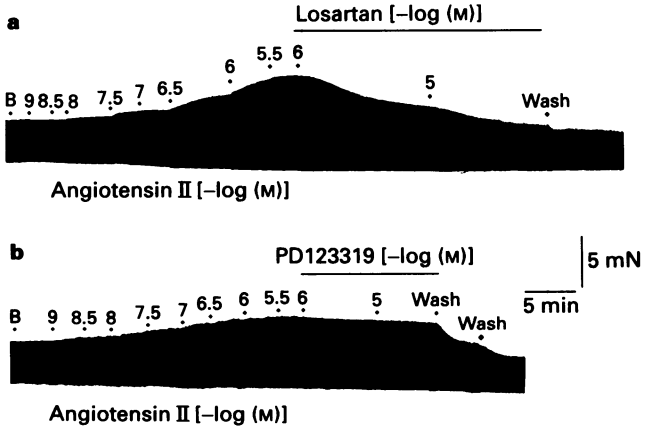

Figure 2 The concentration-dependent effects of angiotensin II (AII 1 μM) on the force of contraction in the presence of 0.3 μM bupranolol and 0.1 μM prazosin and the influence of losartan and PD123319 on the positive inotropic effect of AII in isolated right ventricular papillary muscles from the rabbit. (a) Losartan (1 and 10 μM) was applied when the tension developed by exposure to AII had reached a maximum. (b) PD123319 (1 and 10 μM) was applied when the AII-induced PIE had reached a maximum. B, Basal force of contraction.

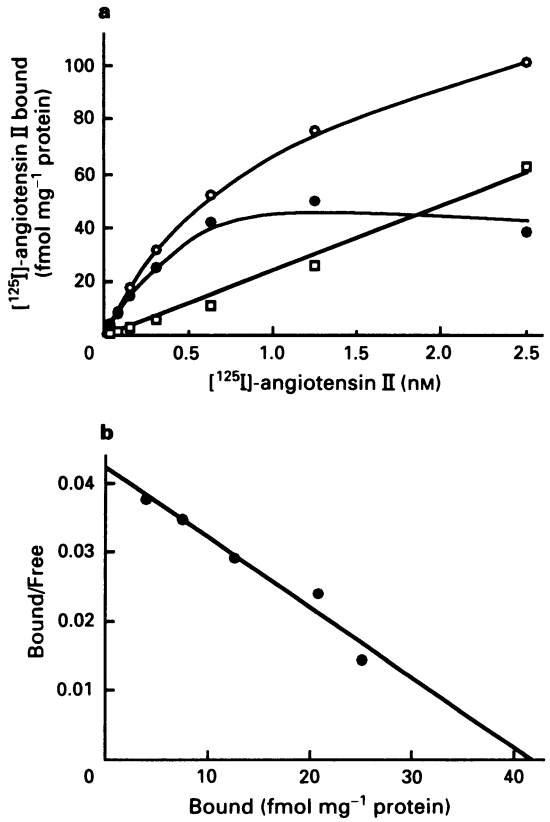

Figure 3 (a) Curves for the saturation binding of [^{125}I]-angiotensin II ([^{125}I]-AII) to the membrane fraction derived from the right ventricular myocardium of the rabbit. Specific binding (●) was defined as total binding (○) minus nonspecific binding (□). (b) Scatchard plot of data calculated from the values in (a). The slope of the regression line was determined by the least-squares methods. Values presented are means of duplicate determinations in a representative experiment.

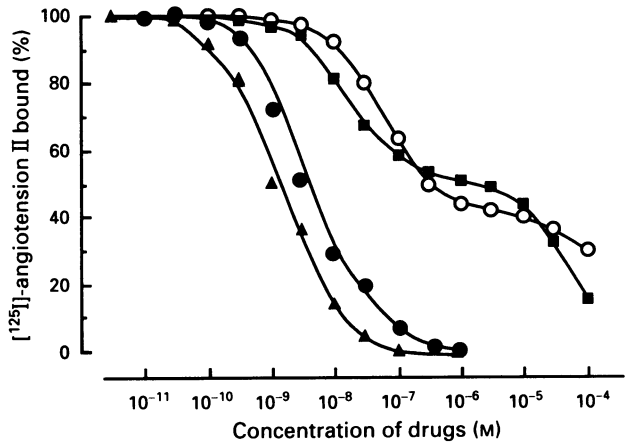

Figure 4 Competition for binding to rabbit ventricular membranes between [^{125}I]-angiotensin II ([^{125}I]-AII, 0.25 nM) and unlabelled AII and non selective and selective antagonists of AII. (●) AII; (▲) saralasin; (■) losartan; (○) PD123319.

receptors in the rabbit ventricular muscle, respectively (Table 2).

Binding experiments with a similar protocol were carried out with membrane fractions from ventricular muscles of the rat, dog and ferret. Saturable high-affinity binding sites for [^{125}I]-AII were found in all of these species. Scatchard analysis of the data indicated the presence of a single class of binding sites in each case. The B_{max} and K_d values for [^{125}I]-AII with membrane fractions from the various species are listed in Table 1. B_{max} values for AII receptors in ventricular muscles from the rabbit, rat and ferret were similar, but the B_{max} value for the dog was significantly higher than the value for the rat (Table 1). The K_d values for [^{125}I]-AII with membrane fractions derived from ventricular muscles of these species were not significantly different from each other when evaluated by ANOVA.

The relative proportions of the subtypes of the AII receptor in the membrane fractions from these species were analysed in terms of displacement of [^{125}I]-AII (0.25 nM) by losartan and PD123319 (Figure 6). In the membrane frac-

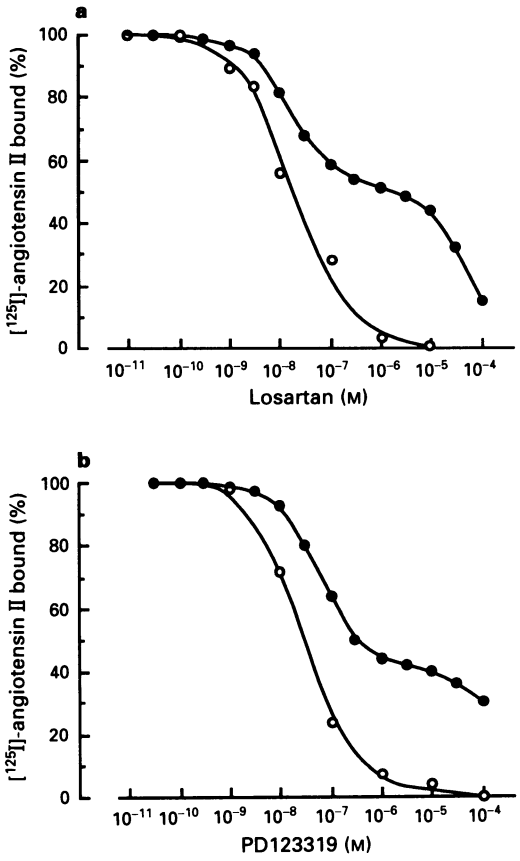

Figure 5 (a) Displacement of [^{125}I]-angiotensin II ([^{125}I]-AII, 0.25 nM) from rabbit ventricular membranes by losartan in the absence (●) and in the presence (○) of PD123319 (10 μM). Binding of [^{125}I]-AII was decreased by PD123319 to 53% of the value in its absence. (b) Displacement of [^{125}I]-AII (0.25 nM) from rabbit ventricular membranes by PD123319 in the absence (●) and in the presence (○) of losartan (1 μM). Binding of [^{125}I]-AII was decreased by losartan to 45% of the value in its absence.

Table 1 Dissociation constants (K_d) and the maximum numbers of binding sites (B_{max}) for [^{125}I]-angiotensin II in membrane fractions derived from the ventricular myocardium of the rabbit, dog, rat and ferret

	Rabbit	*Dog*	*Rat*	*Ferret*
n	4	4	3	4
K_d (nM)	0.97 ± 0.12	1.90 ± 0.20	1.46 ± 0.21	1.51 ± 0.11
B_{max} (fmol mg^{-1} protein)	37.3 ± 5.03	76.1 ± 13.9*	20.7 ± 2.31	37.0 ± 4.55

All values represent means ± s.e.mean in duplicate determinations; *n*, numbers of experiments. *$P < 0.05$ *vs.* the corresponding value in the rat.

Table 2 The binding affinity of losartan and of PD123319 and the relative proportions of the two subtypes of angiotensin II receptor in membrane fractions derived from the ventricular myocardium of the rabbit, dog, rat and ferret

		Losartan-sensitive sites		*PD123319-sensitive sites*		
Species	*PIE*	*pKi*	%	pKi	%	*n*
Rabbit	+	7.89 ± 0.11	46.2 ± 4.67	7.13 ± 0.17	54.2 ± 1.56	4
Dog	–	6.91 ± 0.04	>90	ND	ND	4
Rat	–	7.74 ± 0.46	>90	ND	ND	3
Ferret	–	6.90 ± 0.05	>90	ND	ND	4

All values represent means ± s.e.mean in duplicate determinations; *n*, numbers of experiments; PIE, positive inotropic effect of angiotensin II; ND, not detectable.

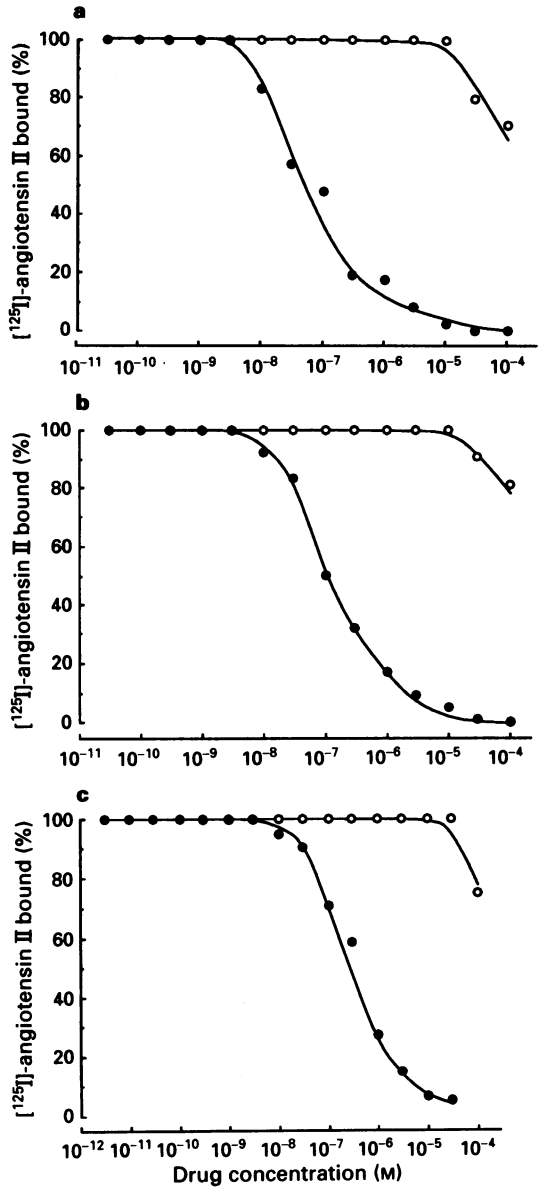

Figure 6 Displacement of [^{125}I]-angiotensin II ([^{125}I]-AII 0.25 nM) from ventricular membrane fractions from (a) the dog, (b) rat and (c) ferret by selective antagonists of AII, PD123319 (○) and losartan (●).

tions derived from ventricular muscles of the rat, dog and ferret, losartan displaced specifically bound [^{125}I]-AII monophasically in a concentration-dependent manner and with high affinity, whereas PD123319 did not displace the binding with high affinity (up to 10 μM) in these species (Figure 6 and Table 2).

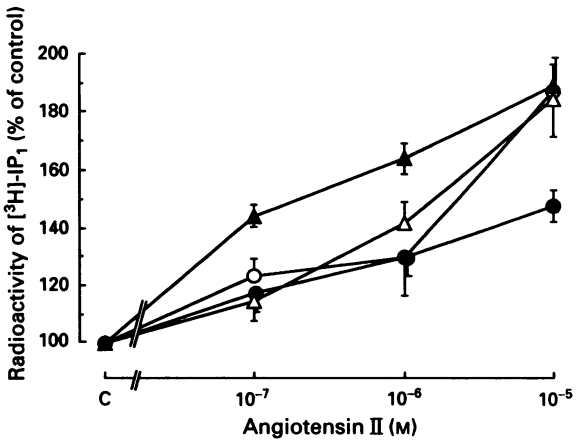

Figure 7 The angiotensin II (AII)-induced accumulation of [^{3}H]-IP$_1$ in ventricular slices from the rabbit (▲), rat (●), dog (○) and ferret (△) that had been prelabelled with *myo*-[^{3}H]-inositol. Concentration-response curves for the accumulation of [^{3}H]-IP$_1$ were generated from data observed 30 min after the addition of AII in the presence of 10 mM LiCl, 0.3 μM bupranolol and 0.1 μM prazosin. Each value indicates the mean ± s.e.mean of results from four or five experiments. C, Control.

Effects of angiotensin II on the hydrolysis of phosphoinositide in ventricular muscles from different mammalian species

Concentration-response curves for the effects of AII on the accumulation of [^{3}H]-IP$_1$ were generated in slices of ventricular muscles from the dog, rat, ferret and rabbit, and the results are presented in Figure 7. Concentration-dependent increases in the accumulation of [^{3}H]-IP$_1$ were induced by AII (0.1–10 μM) in muscle slices isolated from each species. The maximal response to AII in terms of accumulation of [^{3}H]-IP$_1$ in rat ventricles (148.1 ± 5.4%; $n = 5$) was significantly smaller than that in the rabbit. At concentrations of 0.1 and 1 μM, the increase in the accumulation of [^{3}H]-IP$_1$ in response to AII in the rabbit was significantly higher than the increase in other species. However, at 10 μM AII, the increases in the accumulation of [^{3}H]-IP$_1$ were identical in the rabbit, dog and ferret (Figure 7).

Discussion

A wide range of species-dependent variations is apparent in the inotropic effect of AII in mammalian cardiac muscle (Dempsey *et al.*, 1971; Moravec *et al.*, 1990; Ishihata & Endoh, 1993). The PIE of AII was observed only in rabbit ventricular muscle among the mammalian species that we examined in this study. While the reasons for the species-dependent differences in the AII-induced inotropic effect have not been determined, various aspects of the signal-transduction process, including the density of receptors, the

relative proportions of subtypes, second messengers and the subsequent steps that lead to the functional modulation, may be involved. Receptors for AII have been demonstrated in the cardiac muscles of various animals, including the rabbit, chicken, cat and man (Wright *et al.*, 1983; Baker *et al.*, 1984; Rogers, 1984; Rogers *et al.*, 1985, Aceto & Baker, 1990; Moravec *et al.*, 1990). Consistent with these previous findings, saturable high-affinity binding sites for [^{125}I]-AII comparable to those in the rabbit ventricle were found in membrane fractions of rat, dog and ferret ventricular muscles, but AII did not have a PIE in these species. These results indicate that the absence of AII receptors is not the reason for the inability of AII to exert an inotropic effect in these species.

In the rabbit ventricular myocardium, the PIE of AII was inhibited by losartan but was unaffected by PD123319 (Scott *et al.*, 1992; Ishihata & Endoh, 1993). The pK_i value for losartan for displacement of the specific binding of [^{125}I]-AII to the membrane fraction of rabbit ventricle was 7.89, which coincided with the pA_2 value (8.00) for losartan when it antagonizes the PIE of AII. This result provides a further indication that activation of the AT_1 subtype of receptor plays a crucial role in the induction of the PIE of AII in the rabbit. The AT_1 subtype of receptor accounts for 50% of the entire population of AII receptors in the rabbit ventricular myocardium and the other 50% are of the AT_2 subtype that can bind PD123319 with high affinity (Whitebread *et al.*, 1989; Rogg *et al.*, 1990). The latter subtype does not appear to be involved in regulation of myocardial contractility in the rabbit heart (Scott *et al.*, 1992; Ishihata & Endoh, 1993).

It appears crucial to determine whether the AII receptors in the ventricular muscles of the other species in which AII did not produce a PIE are of the AT_1 or the AT_2 subtype. By contrast to the findings in rabbit ventricular myocardium, losartan but not PD123319 displaced specifically bound [^{125}I]-AII with high affinity for the membrane fractions derived from rat, dog and ferret (Table 2 and Figure 6), indicating that the AII receptors in these species are mostly of the AT_1 subtype. These findings imply that neither the AII receptor density nor the distribution of its subtypes accounts for the species-dependent variation of the AII-induced PIE.

Activation of AT_1 receptors is coupled with acceleration of the hydrolysis of phosphoinositide in guinea-pig and rabbit cardiac muscle (Baker & Singer, 1988; Ishihata & Endoh, 1993). We examined, therefore, the effect of AII on the hydrolysis of phosphoinositide in ventricular myocardium of other species in which AII did not elicit a PIE. We found that AII accelerated the hydrolysis of phosphoinositide with comparable potency and efficacy in all of the tested species (Figure 7). Hence, the acceleration of the hydrolysis of phosphoinositide induced by AII is dissociated from the PIE. These findings are in strong contrast to the previous observations, in the rabbit ventricular myocardium, of a close correlation between the hydrolysis of phosphoinositide and the PIE induced via receptors for endothelin, AII and α_1-adrenoceptors (Takanashi *et al.*, 1991; Takanashi & Endoh, 1991; 1992; Ishihata & Endoh, 1993).

Therefore, while the present findings revealed that the dissociation of the acceleration of the hydrolysis of phosphoinositide from PIE might be responsible for the species-dependent variations in the mammalian ventricular myocardium, the reason for dissociation of the PIE from the AII-induced acceleration of the hydrolysis of phosphoinositide in the rat, dog and ferret ventricular myocardium remains to be determined. Although the functional relevance of the acceleration of the hydrolysis of phosphoinositide in mediating the PIE of AII, as well as that of α_1-agonists and endothelin, has not yet been established, evidence has currently been accumulating to indicate that the process that includes activation of protein kinase C by diacylglycerol, as well as inositol 1,4,5-trisphosphate (IP_3), the product of phosphoinositide hydrolysis, may lead to modulation of Ca^{2+} signalling in both the facilitatory and the inhibitory direction. Since the coupling of receptors to these divergent transduction processes, which include Ca^{2+} channels, different types of K^+ channel, the Na^+/H^+ exchanger, Na^+, K^+- ATPase and contractile proteins, appears to vary markedly depending on the species (Endoh, 1991), we postulate that the difference in the extent of the coupling with facilitatory and inhibitory pathways that are triggered by activation of AII receptors may be crucial for determination of the final inotropic response to AII of individual species.

Dissociation of PIE from acceleration of the hydrolysis of phosphoinositide has been reported previously. While AII accelerated the hydrolysis of phosphoinositide in both atrial and ventricular muscle, it produced a PIE via AT_1 receptors only in atrial (Feolde *et al.*, 1993) and not ventricular muscle (Baker *et al.*, 1992). It has also been shown that AII activates phospholipase D as well as phospholipase C (Sadoshima & Izumo, 1993). In rat smooth muscle, AII has been shown to activate Na^+/H^+ exchange in association with the acceleration of the hydrolysis of phosphoinositide via activation of AT_1 receptors (Berk *et al.*, 1987; Ko *et al.*, 1992), but such results from cardiac muscle are still lacking at present. The physiological relevance of the AT_2 subtype of receptor in regulation of cardiac function is unknown, but various functional roles for this subtype have been proposed in other cellular systems. For example, AT_2 receptors may be involved in handling of water in the kidney (Keiser *et al.*, 1992), synthesis of prostaglandin in human astrocytes (Jaiswal *et al.*, 1991), as well as in increases in transient K^+ and delayed-rectifier K^+ currents (Kang *et al.*, 1993) and in decreases in levels of cyclic GMP (Summers *et al.*, 1991) in rat cultured neurones. Although the AT_2-receptor antagonist PD123319 displaced AII from its binding sites, it affected neither the basal force nor the AII-induced PIE. Since pretreatment with PD123319 and with another AT_2 antagonist, PD121981 (Scott *et al.*, 1992), did not affect the inhibition by losartan of the AII-induced PIE, there may be no functionally relevant mutual or allosteric interaction between AT_1 and AT_2 receptors in the regulation of cardiac contractility. Therefore, it is not considered likely that the sparse distribution of AT_2 receptors in the rat, dog and ferret ventricular myocardium is responsible for the absence of a PIE of AII in these species.

In summary, the AT_1 subtype of receptor for AII is present in ventricular muscles of the rat, dog and ferret, in which AII does not elicit a PIE. Since AII stimulates the hydrolysis of phosphoinositide in these species, we postulate that the dissociation of the production of IP_3 and diacylglycerol from the PIE may be responsible for the species-dependent variations in the AII-induced inotropic actions on mammalian ventricular myocardium.

This study was supported in part by a Grant-in-Aid (no. 05770058) for the Encouragement of Young Scientists (A) and by a Grant-in-Aid (no. 03253102) for the Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan. The authors are grateful to Du Pont, Pharmaceuticals & Biotechnology R & D Delaware, U.S.A. for a generous supply of the selective AT_1-receptor antagonist losartan; to Dr Joan A. Keiser, Warner-Lambert Co., Ann Arbor, MI, U.S.A, for a generous supply of the selective AT_2-receptor antagonist PD123319; to Pfizer Taito, Tokyo, for prazosin; and to Kaken Pharmaceutical Co., Tokyo, for (±)-bupranolol.

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(Received June 23, 1994
Accepted September 6, 1994)