Evidence for the involvement of different receptor subtypes in the pre- and postjunctional actions of angiotensin II at rat sympathetic neuroeffector sites

S.L. Cox, *A. Ben, D.F. Story, & ¹J. Ziogas

Pharmacology Unit, Department of Medical Laboratory Science, RMIT, Melbourne, Victoria, Australia and

*Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia

1 The effects of the nonpeptide angiotensin II receptor (AT) antagonists losartan and PD 123319 on actions of angiotensin II in the rat caudal artery and rat vas deferens preparations were investigated.

2 Angiotensin II (1.0 μM) increased perfusion pressure in isolated segments of the rat caudal artery. This increase in perfusion pressure was prevented by the AT₁-antagonist, losartan (0.1 μM) but was not affected by the AT₂-antagonist, PD 123319 (0.1 μM).

3 Angiotensin II (0.1–3.0 μM) produced a concentration-dependent enhancement of the stimulation-induced (S-I) efflux of [³H]-noradrenaline from isolated segments of rat caudal artery in which the noradrenergic transmitter stores had been labelled with [³H]-noradrenaline. The maximum enhancement of S-I efflux was approximately 60% with 1.0 μM angiotensin II.

4 Losartan (0.01 and 0.1 μM) reduced the enhancement of S-I efflux produced by 1.0 μM angiotensin II in the caudal artery.

5 PD 123319 (0.01 μM) did not affect the enhancement of S-I efflux produced by angiotensin II (1.0 μM) in the caudal artery. However, in a higher concentration (0.1 μM), PD 123319 reduced the enhancement of S-I efflux produced by 1.0 μM angiotensin II.

6 Angiotensin II produced concentration-dependent enhancement of the purinergic twitch responses (1 pulse/60 s) in the rat vas deferens,

7 Losartan (0.03 μM) and PD 123319 (0.03 μM) each reduced the angiotensin II-induced enhancement of the twitch responses in the rat vas deferens.

8 These findings indicate that the enhancement of sympathetic neuroeffector transmission in both the caudal artery and vas deferens of the rat involves angiotensin receptor subtype(s) sensitive to both losartan and PD 123319. In contrast, the direct vasoconstrictor effect of angiotensin II in the rat caudal artery involves activation of a receptor subtype sensitive only to losartan.

Keywords: Angiotensin II; losartan; PD 123319; vasoconstriction; sympathetic neuroeffector transmission

Introduction

The renin-angiotensin system plays a central role in cardiovascular homeostasis by influencing vascular tone, extracellular fluid and electrolyte balance, and the sympathetic nervous system (Sealey & Laragh, 1989). The interactions of the renin-angiotensin system with the cardiovascular system are predominantly mediated by the octapeptide, angiotensin II. Angiotensin II influences cardiovascular function by several mechanisms, including direct constriction of resistance and capacitance vessels and direct cardiac inotropic and chronotropic activity (Sealey & Laragh, 1989). In addition, angiotensin II has been shown to facilitate noradrenergic neuroeffector transmission by enhancing stimulation-induced release of noradrenaline from sympathetic nerves, although it has also been reported to increase the rate of synthesis of noradrenaline and to inhibit neuronal uptake of the transmitter (Story & Ziogas, 1987). More recently, angiotensin II has been shown to modulate the release of co-transmitters from sympathetic nerve terminals. Thus, in the guinea-pig vas deferens, with low frequencies of stimulation, angiotensin II enhances noradrenergic and purinergic transmission (Ellis & Burnstock, 1989; Ziogas & Cunnane, 1991). With higher frequencies of stimulation, although the noradrenergic component is still enhanced, the purinergic component is not affected (Ellis & Burnstock, 1989). Trachte (1988a) reported

that, in the rabbit vas deferens, angiotensin II produces the usual enhancement of noradrenergic transmission, but that the peptide inhibits the purinergic component.

The receptors through which angiotensin II acts to produce its diverse effects were considered homogeneous; however, in the past five years evidence has been obtained for the existence of distinct receptor subtypes (Whitebread *et al.*, 1989; Chiu *et al.*, 1989; Smith *et al.*, 1992). This advance was stimulated by the development of non-peptide receptor antagonists such as DuP 753 (now known as losartan), and PD 123319 and the related PD 123177. Based on numerous radioligand receptor binding and autoradiographic studies, angiotensin receptors are now divided into two major subtypes, subtype 1 (AT₁) and subtype 2 (AT₂). The angiotensin II receptor subtype sensitive to losartan has been designated AT₁, while the receptor sensitive to either PD 123319 or PD 123177 has been designated AT₂. Recent cloning studies suggest that there are two subtypes of AT₁ receptors; termed AT_{1A} and AT_{1B} (Iwai & Inagami, 1992; Murphy *et al.*, 1991).

In functional studies *in vivo* and *in vitro*, the AT₁ receptor antagonist, losartan, effectively attenuates many actions of angiotensin II, including contraction of isolated vascular and nonvascular smooth muscle and cardiac muscle (Smith *et al.*, 1992), stimulation of aldosterone and catecholamine release, its dipsogenic effect, and potentiation of responses to sympathetic nerve stimulation (Wong *et al.*, 1990a). The AT₂ antagonists PD 123177 and PD 123319 are generally without

¹ Author for correspondence at: Department of Medical Laboratory Science, RMIT, GPO Box 2476V, Melbourne 3001, Australia.

effect. Therefore, despite overwhelming evidence for two major subtypes of receptors, the functional responses to angiotensin II seem to be mediated exclusively by activation of the AT₁ receptor. An important physiological consequence of angiotensin II binding to AT₂ receptor sites remains to be established.

In the present study, the non-peptide angiotensin II receptor antagonists, losartan and PD 123319, were used to characterize the receptor subtypes through which angiotensin II mediates its effects on sympathetic neuroeffector function in the rat isolated caudal artery and vas deferens.

Methods

Rat caudal artery preparations

Sprague-Dawley rats (250–350 g) of either sex were killed by a blow to the head and exsanguinated. A segment of the central caudal artery (3–4 cm) was dissected free from the connective tissue and transferred to physiological salt solution (PSS) which had been bubbled with 95% O₂ and 5% CO₂ and maintained at 37°C. Artery segments (1–2 cm) were cannulated at the proximal end and mounted longitudinally under 0.5 g tension, with the proximal end lowermost. The distal end was tied off and a small incision was made through the artery wall, close to the upper tie around the distal end to enable the perfusate to superfuse the adventitial surface. The segments were perfused and superfused at a constant flow rate at 4 ml min⁻¹ with PSS by use of a Gilson Minipuls-2 peristaltic pump. Luminal perfusion pressure was monitored with a Grass P23 I.D. blood pressure transducer (Gould Inc., Oxnard, California, U.S.A.) and recorded on a Macintosh IIci computer using a MacLab/4 data acquisition system and chart v3.2.6. software (Analogue Digital Instruments, Sydney, Australia). The A/D sampling was usually between 250–1000 Hz. Circular, bipolar, platinum electrodes were placed around the segments to enable periarterial sympathetic nerve stimulation. Artery segments were allowed to equilibrate for 15 min before the start of experimental procedures.

In the first series of experiments, 20 min after the initial equilibration period, the vasoconstrictor responses to angiotensin II were investigated by introducing 1.0 µM angiotensin II into the PSS perfusing/superfusing the artery preparations. Each artery preparation was exposed only once to angiotensin II since preliminary experiments demonstrated that there was marked desensitization to the peptide with second and subsequent exposures. In experiments in which the effect of losartan and PD 123319 on vasoconstrictor responses to angiotensin II were investigated, the antagonists were introduced to the perfusion fluid in a concentration of 0.1 µM, 20 min before the introduction of angiotensin II.

In the second series of experiments, after the 15 min equilibration period artery segments were stimulated (5 Hz, 10 s) to assess their viability. Suitable preparations were then removed from the organ baths and incubated for 30 min in 2 ml of PSS containing [³H]-noradrenaline (10 µCi ml⁻¹, 0.67 µM). After incubation with [³H]-noradrenaline, artery segments were again set up for perfusion and superfusion, at a constant flow rate of 4 ml min⁻¹, with PSS. After 60 min of perfusion and superfusion, a 30 s train of 1 ms square wave pulses at a frequency of 5 Hz was applied to the preparations in order to facilitate the removal of non-specifically bound radioactivity from the tissues. After a further 30 min of perfusion/superfusion, the artery segments were subjected to two periods of electrical field stimulation (5 Hz, 30 s), delivered 30 min apart. In each stimulation period, 6 consecutive 1-min collections (4 ml volume) of the perfusate/superfusate solution were taken for measurement of the efflux of [³H]-noradrenaline from the artery segments. In each period, stimulation was given after the second collection. In experiments in which the effects of angiotensin II and non-

peptide angiotensin II antagonists were investigated, on resting and S-I effluxes of [³H]-noradrenaline, these drugs were introduced to the perfusion/superfusion fluid 20 min before the second period of stimulation.

The perfusate/superfusate was collected in ice-cold glass vials containing 10 µl Na₂SO₃ (1 µM) and 10 µl ethylenediaminetetraacetic acid disodium salt (0.3 M) to limit oxidation of the catecholamines. These samples were frozen for subsequent chromatographic separation of [³H]-noradrenaline and its tritiated metabolites.

Separation of [³H]-noradrenaline from its metabolites

[³H]-noradrenaline and its metabolites were separated by column chromatography with alumina and Dowex -50W ion-exchange resin, using a method adapted from that of Graefe *et al.* (1973). The pH of each collection of perfusate/superfusate was adjusted to pH 8.3–8.5 with 1 M Tris-acetate buffer pH 8.7. Each sample was first passed through an alumina column which adsorbed the catechol compounds noradrenaline (NA) and 3,4-dihydroxyphenylethylene-glycol (DOPEG). NA and DOPEG were then eluted from the alumina columns with two 1 ml aliquots of acetic acid (0.4 M) and NA was then separated from DOPEG by adsorption on Dowex -50W columns. NA was eluted from the Dowex -50W columns with two 0.5 ml aliquots of 6 M HCl/ethanol (1:1, v/v). To determine the recovery of [³H]-noradrenaline from the columns, a known quantity of purified [³H]-noradrenaline was routinely passed through the columns. The values of resting and stimulation-induced effluxes given have not been corrected for recovery (73 ± 2.9%).

Determination of [³H]-noradrenaline

Fractions (1 ml) eluted from the Dowex -50W columns with HCl/ethanol (see above) were mixed with 12 ml of scintillation fluid (Packard, Ultima Gold XR) and the radioactivity present was determined by liquid scintillation counting (Packard Tri-carb 2000). Corrections for counting efficiency were made by external automatic standardisation and the results expressed as disintegrations per minute (d.p.m.).

The resting efflux (R₁ and R₂) of [³H]-noradrenaline preceding each of the two periods of stimulation was determined as the mean content of [³H]-noradrenaline in the two 1-min collections of the perfusate/superfusate solution taken immediately before each period of stimulation. The S-I efflux of [³H]-noradrenaline for each stimulation period was calculated by subtracting the corresponding resting efflux from the content of [³H]-noradrenaline in each of the four consecutive 1-min collections of superfusate, taken from the onset of stimulation, if the radioactivity present exceeded the mean resting efflux, and summing the differences. In each experiment, the resting and S-I effluxes of [³H]-noradrenaline for the second period of stimulation were each expressed as a percentage of their corresponding value for the first period of stimulation, (% R₂/R₁) and (% S₂/S₁), respectively.

Rat vas deferens preparation

Male Sprague-Dawley rats (250–350 g) were killed by decapitation. Whole vasa deferentia were removed and carefully stripped of connective tissue and blood vessels. Segments of 1–2 cm were dissected from the prostatic end and placed longitudinally in tissue baths. A pair of platinum ring electrodes was placed around the prostatic end of each preparation and the epididymal end was attached to a Ugo Basile isometric transducer. The preparations were perfused at 2 ml min⁻¹ with PSS by use of a Gilson Minipuls-2 peristaltic pump. An initial resting tension of 1 g was added to each preparation and changes in tension were recorded on a Rikadenki double channel pen recorder. The preparations were allowed to equilibrate for 30 min.

After the equilibration period, the preparations were stimulated electrically with 1 pulse every 60 s. The voltage was supra-maximal (30 V) and the pulse duration was 0.3 ms. After 10 min of stimulation the responses had stabilized, at which time the PSS solution was changed to a solution containing angiotensin II (3–600 nM) and the tissue was then stimulated for a further 10 min. A further 10 responses were obtained after the washout of angiotensin II. The tissue was then perfused without stimulation for 10 min and the above procedures were repeated using the next higher concentration of angiotensin II. In a separate series of experiments, either losartan or PD 123319 was introduced into the PSS after the initial 10 min stimulation period and a series of twitch responses were evoked in the presence of the antagonist over 10–15 min. The effect of angiotensin II on the twitch responses was then re-examined, in the presence of the antagonist, as described above.

Drugs and radiochemicals

The PSS had the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 0.45, NaHCO₃ 25, KH₂PO₄ 1.03 and D-(+)-glucose 11.1. Ethylenediaminetetraacetic acid disodium salt (0.067 M) and ascorbic acid (0.14 M) were also present to prevent the oxidation of noradrenaline.

The following drugs were used: angiotensin II (synthetic, human sequence, Sigma, U.S.A.); losartan (gift from Du Pont Merck Pharmaceuticals, Wilmington, Delaware), PD 123319 ((S)-1-[[4-dimethylamino]-3-methylphenyl]methyl]-5-(di-phenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid, ditrifluoroacetate, dihydrate; gift from Parke Davis, Ann Arbor, Michigan); prazosin hydrochloride (Pfizer, U.S.A.); and α , β -methylene-adenosine-5'-triphosphate (α , β -meATP) (Sigma, U.S.A.).

Angiotensin II stock solutions were prepared in concentrations of 1 mM in 5% acetic acid and stored in 50 μ l aliquots at 0°C. Stock solutions of losartan and PD 123319 (1.0 mM) were prepared in deionised water and stored at 5°C. Prazosin was prepared by dissolution in deionised water, containing 5% dextrose and 10% glycerol to give a 1 M stock solution. A 1 mM stock solution of α , β -meATP was prepared in deionised water and stored at 0°C. All stock solutions were

diluted in PSS and added to the reservoirs supplying the organ bath.

Tritiated noradrenaline (1-[7,8-³H]-noradrenaline) was supplied by the Radiochemical Centre, Amersham, U.K., with a specific activity of 15 Ci mmol⁻¹ and a radioactive concentration of 1.0 mCi ml⁻¹.

Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (s.e.mean); *n* represents the number of experiments. For the rat caudal artery experiments, groups of means were tested for significant differences by using one-way analysis of variance (ANOVA) and planned comparisons (Student's unpaired *t* test) between predetermined pairs of means. For the concentration-effect curves obtained in the rat vas deferens, analysis of co-variance (ANCOVA) tests were performed with the computer software Regression and Empirical Analytical Procedures (Gamma Research Systems). Data within time-control or angiotensin II concentration-effect groups were tested by ANOVA to determine if there were any time- or concentration-dependent effects. In all cases probability levels less than 0.05 ($P < 0.05$) were taken to indicate significant differences between means.

Results

Effects of angiotensin II on perfusion pressure in the rat caudal artery in the absence and presence of losartan and PD 123319

When isolated caudal artery segments were perfused and superfused at a constant flow rate of 4 ml min⁻¹, the luminal perfusion pressure stabilised to between 20 and 40 mmHg within 10–15 min and, in the absence of drugs, remained stable. Addition of angiotensin II (1.0 μ M) to the perfusion/superfusion solution produced an increase in perfusion pressure of 44 ± 2 mmHg ($n = 6$). The peak increase in perfusion pressure occurred within 20–30 s and the perfusion pressure declined to basal over the next 2–3 min in the continued presence of angiotensin II.

The receptor antagonists, losartan (0.1 μ M) and PD 123319 (0.1 μ M), did not significantly alter the basal perfusion pressure ($P > 0.05$, ANOVA, unpaired *t* tests). The AT₁-antagonist, losartan (0.1 μ M), when introduced into the perfusion/superfusion fluid 20 min before the addition of angiotensin II (1.0 μ M), abolished the vasoconstrictor response to angiotensin II (Figure 1). In contrast, as shown in Figure 1, the vasoconstrictor response to angiotensin II (1.0 μ M) was unaltered by the presence of the AT₂-antagonist PD 123319 (0.1 μ M).

Effects of angiotensin II on the resting and S-I effluxes of [³H]-noradrenaline in the rat caudal artery

Rat caudal artery segments loaded with [³H]-noradrenaline were given two periods of electrical field stimulation (5 Hz, 30 s) at 30 min intervals. In control experiments, the mean resting efflux of [³H]-noradrenaline, preceding the first period of stimulation, was 207 ± 20 d.p.m. ($n = 7$). There was a progressive decline in the resting efflux of [³H]-noradrenaline between the two periods of stimulation. The mean value of the resting efflux of [³H]-noradrenaline preceding the second period of stimulation, expressed as a percentage of that preceding the first (% R_2/R_1) was $83 \pm 6\%$ ($n = 7$). Stimulation of the periaxillary sympathetic nerves (5 Hz, 30 s) of the artery segments produced an increased efflux of [³H]-noradrenaline. The mean absolute stimulation-induced (S-I) efflux of [³H]-noradrenaline evoked by the first period of stimulation was 670 ± 154 d.p.m. ($n = 7$). The mean value of the S-I efflux of [³H]-noradrenaline with the second period of stimulation, expressed as a percentage of that for the first (% S_2/S_1), was $96 \pm 7\%$ ($n = 7$).

Figure 1 The effects of losartan (0.1 μ M) and PD 123319 (0.1 μ M) on the vasoconstrictor response to 1.0 μ M angiotensin II in isolated perfused/superfused rat caudal artery preparations. The antagonists were introduced 20 min prior to addition of angiotensin II. The vertical axis represents increases in perfusion pressure in mmHg. The columns represent the mean increases in perfusion pressure produced by angiotensin II in the absence (Con) and presence of the antagonists losartan and PD 123319. The vertical lines represent the standard errors of the means from 4–8 experiments. Losartan, but not PD 123319 significantly ($P < 0.05$) reduced the vasoconstrictor responses to angiotensin II (ANOVA, unpaired *t* tests).

Introduction of angiotensin II (0.1–3.0 μM) to the perfusion/superfusion fluid, 20 min before the second period of stimulation, did not significantly alter the resting efflux of [^3H]-noradrenaline ($P > 0.05$ ANOVA). In contrast, as shown in Figure 2, angiotensin II (0.1–3.0 μM) produced concentration-dependent increases in the S-I efflux of [^3H]-noradrenaline. The maximum enhancement of S-I efflux of 60–70% was produced by 1.0 μM angiotensin II. Further increases in concentration of angiotensin II actually produced less enhancement (Figure 2).

Effect of losartan on the enhancement of S-I efflux by angiotensin II in the rat caudal artery

Losartan in concentrations of 0.01 and 0.1 μM , added to the perfusion/superfusion fluid 20 min before the second period of stimulation, did not significantly alter the resting or S-I effluxes of [^3H]-noradrenaline ($P > 0.05$, ANOVA). As shown in Figure 3, the enhancement of S-I efflux produced by 1.0 μM angiotensin II was reduced by 0.01 μM losartan and abolished by 0.1 μM losartan.

Effect of PD 123319 on the enhancement of S-I efflux by angiotensin II in the rat caudal artery

PD 123319 in concentrations of 0.01 and 0.1 μM , added to the perfusion/superfusion fluid 20 min before the second period of stimulation, did not significantly alter the resting or S-I effluxes of [^3H]-noradrenaline ($P > 0.05$, ANOVA). As shown in Figure 4, the enhancement of S-I efflux produced by 1.0 μM angiotensin II was not significantly altered by 0.01 μM PD 123319. In contrast, the enhancement of S-I efflux by 1.0 μM angiotensin II was significantly reduced by 0.1 μM PD 123319 (Figure 4).

Effect of angiotensin II on twitch responses of the rat vas deferens evoked by electrical stimulation

Electrical stimulation with 1 pulse every 60 s of prostatic segments of the rat vas deferens generally produced rapid,

Figure 2 Log concentration-response relationship for the effect of angiotensin II on the enhancement of stimulation-induced (S-I) efflux of [^3H]-noradrenaline from rat isolated caudal artery preparations in which the noradrenergic transmitter stores had been radiolabelled with [^3H]-noradrenaline. The periaxillary sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s) at 30 min intervals. In each experiment, the S-I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S₂/S₁). The unbroken horizontal line represents the mean S-I efflux in the absence of angiotensin II and the broken lines the standard error of the mean. The filled circles (●) represent the effects of angiotensin II (0.1–3.0 μM) when introduced to the perfusion fluid 20 min before the second period of stimulation. The points plotted represent the means $\pm$ s.e. means from 4–8 experiments.

monophasic responses. Addition of 1 μM α , β -meATP produced an initial enhancement of these twitch responses, lasting for 4–5 min. However, prolonged exposure desensitized the P₂-purinoceptors and abolished the twitch responses. In some experiments, the responses were biphasic and the effect of the α_1 -adrenoceptor antagonist prazosin, was assessed on these responses. Prazosin, in a concentration of 1 μM , completely abolished the second phase of these biphasic responses, leaving a large twitch response to nerve stimulation. In preliminary experiments, angiotensin II clearly enhanced both phases of the biphasic twitch responses. However, for the present study, the effect of angiotensin II was assessed only on the monophasic purinergic twitch responses.

In 9 experiments, the mean control response prior to the addition of the first concentration of angiotensin II was 1.1 ± 0.2 g. By allowing a 10 min rest period without stimulation after washout of each concentration of angiotensin II (see methods), the responses were well maintained and there were no significant differences between the control responses obtained prior to each addition of angiotensin II ($P > 0.05$, ANOVA). As shown in Figures 5 and 6, in the absence of antagonists, addition of angiotensin II (3–300 nM) produced concentration-dependent enhancement of the twitch responses to nerve stimulation.

Effect of losartan on the enhancement of twitch responses by angiotensin II in the rat vas deferens

When losartan (0.01 and 0.03 μM), was added to the perfusion solution there was an initial enhancement in the twitch responses (10–15%). Once the responses had stabilized, losartan (0.01 and 0.03 μM) produced a concentration-dependent reduction in the enhancement of twitch responses produced by angiotensin II (Figure 5).

Figure 3 Effect of 1.0 μM angiotensin II in the absence and presence of losartan on the stimulation-induced (S-I) efflux of radioactive noradrenaline from [^3H]-noradrenaline-labelled rat caudal artery preparations. The periaxillary sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s), at 30 min intervals. In each experiment, the S-I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S₂/S₁). Angiotensin II, losartan, or a combination of angiotensin II and losartan (0.01 or 0.1 μM), was introduced 20 min before the second period of stimulation. Open columns represent the respective control (Con) experiments and the hatched columns, experiments in which angiotensin II (All) was added in either the absence or presence of losartan. Columns represent the means $\pm$ s.e. means from 4–6 experiments. *Significant differences cf. respective control ($P < 0.05$, ANOVA, unpaired t tests).

Figure 4 Effect of 1.0 μM angiotensin II in the absence and presence of PD 123319 on the stimulation-induced (S-I) efflux of radioactive noradrenaline from [³H]-noradrenaline-labelled rat caudal artery preparations. The periarterial sympathetic nerves were stimulated for 2 periods (5 Hz, 30 s), at 30 min intervals. In each experiment, the S-I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S₂/S₁). Angiotensin II, PD 123319, or a combination of angiotensin II and PD 123319 (0.01 or 0.1 μM), was added 20 min before the second period of stimulation. Open columns represent the respective control (Con) experiments and the hatched columns, experiments in which angiotensin II (All) was added in either the absence or presence of PD 123319. Columns represent the means ± s.e.means from 4–6 experiments. *Significant differences cf. respective control ($P < 0.05$, ANOVA, unpaired t tests).

Effect of PD 123319 on the enhancement of twitch responses by angiotensin II in the rat vas deferens

Exposure of the tissue to PD 123319 had no significant effect on the twitch responses to nerve stimulation ($P < 0.05$, t test). As shown in Figure 6, PD 123319 (0.01 and 0.03 μM), reduced the enhancement of twitch responses by angiotensin II in a concentration-dependent manner.

Discussion

Inhibition of the vasoconstrictor action of angiotensin II by the AT₁-antagonist losartan has been demonstrated in various isolated tissue preparations, including rabbit aorta and uterus (Dudley *et al.*, 1990), rat pulmonary artery (Chang & Lotti, 1989) and rat portal vein (Rhaleb *et al.*, 1991). Similarly, in the present study, in the rat caudal artery, the increase in perfusion pressure produced by angiotensin II was blocked by losartan and not affected by PD 123319. Such *in vitro* findings are consistent with those of *in vivo* studies which have demonstrated that increases in blood pressure produced by angiotensin II are mediated through AT₁ receptors (Wong *et al.*, 1990b). Whereas it is clear that the postjunctional vasoconstrictor effect of angiotensin II is subserved by AT₁ receptors, the receptor(s) involved in the action of angiotensin II on transmitter noradrenaline release is not as well defined.

In rat isolated caudal artery preparations, in which transmitter stores had been radiolabelled with [³H]-noradrenaline, angiotensin II produced concentration-dependent enhancement of the S-I efflux of [³H]-noradrenaline. The maximum

Figure 5 Pooled data showing enhancement of single pulse twitch responses of rat vas deferens preparations by angiotensin II (○), and inhibition of these responses by losartan 0.01 μM (□) and 0.03 μM (Δ) ($P < 0.05$, ANCOVA). The points plotted represent means with s.e.means of 4–6 experiments.

Figure 6 Pooled data showing enhancement of single pulse twitch responses of rat vas deferens preparations by angiotensin II (○), and inhibition of these responses in the presence of PD 123319 0.01 μM (□) and 0.03 μM (Δ) ($P < 0.05$, ANCOVA). The points plotted represent means of 4–6 experiments ± s.e.means.

enhancement occurred with 1 μM angiotensin II, and further increases in concentration to 3 μM produced less enhancement. Such a 'bell shaped' concentration-effect relationship has also been reported for the modulation of sympathetic transmission by angiotensin II in guinea-pig atria (Ziogas *et al.*, 1984) and rabbit vas deferens (Trachte *et al.*, 1990; Hedge & Clarke, 1993). The enhancement of S-I [³H]-noradrenaline efflux by angiotensin II was blocked by losartan (0.01 and 0.1 μM) and by PD 123319 (0.1 μM). Similar findings were also observed in the rat vas deferens where angiotensin II produced a concentration-dependent enhancement of the twitch responses to sympathetic nerve stimulation. This enhancement was also blocked by both losartan and PD 123319. These findings suggest that the prejunctional angiotensin II receptor has different characteristics from the postjunctional receptor.

Previous studies in which the nature of the prejunctional angiotensin II receptor has been investigated, have given rise to differing conclusions. Several reports suggest that the prejunctional receptor is of the AT₁ subtype. For example, in

guinea-pig isolated atria, the enhancement of noradrenaline release by angiotensin II was blocked by losartan but unaffected by PD 123319 (Brasch *et al.*, 1993). Similarly, the enhancement of noradrenergic transmission in the rat mesenteric vascular bed by angiotensin II was abolished by losartan (Parrish & Cassis, 1991; Tofovic *et al.*, 1991) but not influenced by PD 123177 (Tofovic *et al.*, 1991). The prejunctional enhancement of renal noradrenergic function in the anaesthetized dog was also blocked by losartan (Wong *et al.*, 1990c). In contrast, in the rabbit isolated vas deferens, both losartan and PD 123177 failed to inhibit the enhancement of noradrenergic transmission by angiotensin II (Trachte *et al.*, 1990; 1991). Thus, Trachte *et al.* (1991) suggested that neither AT₁ nor AT₂ receptors are involved in the enhancement of noradrenergic transmission by angiotensin II. In the present study, the enhancement by angiotensin II, of noradrenergic transmission in the rat caudal artery, was inhibited by both losartan and PD 123319, suggesting that the prejunctional receptor differs from the major AT₁ and AT₂ subtypes.

Several studies have suggested that the angiotensin II receptors involved in modulation of sympathetic noradrenergic and purinergic transmission in vas deferens preparations are not homogeneous. Thus, in the guinea-pig vas deferens, with 2 Hz stimulation, angiotensin II enhanced both noradrenaline and ATP release, but at 20 Hz stimulation, only noradrenaline release was enhanced by angiotensin II (Ellis & Burnstock, 1989). The heptapeptide angiotensin III enhanced noradrenaline release, but inhibited ATP release; however, no antagonists were used to determine the subtypes of angiotensin receptors involved in these actions of angiotensin II and angiotensin III (Ellis & Burnstock, 1989). In the rabbit vas deferens, angiotensin II and angiotensin III each enhanced the noradrenergic component of the biphasic response to sympathetic nerve stimulation and inhibited the purinergic component (Trachte, 1988a). Losartan did not prevent the enhancement of the noradrenergic component but it did prevent the inhibition of the purinergic component by angiotensin II and also the release of prostaglandin E evoked by angiotensin II (Trachte *et al.*, 1990). In the guinea-pig vas deferens, angiotensin II usually enhanced purinergic excitatory junction potentials; however, following reserpine treatment, angiotensin II inhibited purinergic transmission (Ziogas & Cunnane, 1991). Interestingly, the inhibition was prevented by pretreatment with the cyclo-oxygenase inhibitor, indomethacin (Ziogas & Cunnane, 1991). Several studies have demonstrated that endogenously released prostaglandins can interfere with the prejunctional enhancement of sympathetic transmission by angiotensin II in a number of preparations. Thus, Lanier & Malik (1982) demonstrated that the enhancement of noradrenaline release in the rat heart by angiotensin II was attenuated by prostaglandins released from the myocardium. In the rabbit ear artery the inhibition of sympathetic neurotransmission by angiotensin II was reversed by indomethacin pretreatment (Rónai, 1991). Trachte (1988b) reported that indomethacin and eicosatetraenoic acid, but not acetylsalicylic acid, could prevent the inhibition of purinergic transmission by angiotensin II in the rabbit vas deferens. Therefore, it is likely that the inhibition of the purinergic response by angiotensin II in the rabbit vas deferens may involve stimulation of a postjunctional AT₁ receptor which subserves release of prostaglandins. An

inhibitory action involving prostaglandins does not appear to interfere with the enhancement of purinergic transmission reported in this study with the rat vas deferens or in the guinea-pig vas deferens (Ellis & Burnstock, 1989). Whether these differences relate to species or differences in other experimental conditions remains to be determined. None the less, the findings of this study indicate that the enhancement of sympathetic purinergic transmission by angiotensin II may involve a prejunctional receptor that is sensitive to both losartan and PD 123319.

As mentioned in the Introduction, the original studies on which the AT₁ and AT₂ classifications were based, indicated that AT₂ antagonists are generally ineffective in blocking the multitude of actions of angiotensin II in the cardiovascular system (Smith *et al.*, 1992). However, recent studies have demonstrated that the AT₂ antagonist used in the present study, PD 123319, can inhibit the actions of angiotensin II in certain preparations. Thus, Kunert-Radek *et al.* (1994) have recently shown that the stimulatory effect of angiotensin II on the proliferation of mouse spleen lymphocytes was blocked by both losartan and PD 123319. Similarly, the intracerebroventricular injection of angiotensin II in anaesthetized rats (Widdop *et al.*, 1993a) and the angiotensin II-mediated drinking response in rats (Rowland *et al.*, 1992) are both blocked by PD 123319. In contrast, the related AT₂ antagonist, PD 123177, was without effect on the intracerebroventricular injection of angiotensin II (Widdop *et al.*, 1993b; Rowland *et al.*, 1992). Therefore, it is likely that PD 123319 is not as selective for the AT₂ receptor as initially reported and that functional subtypes of the AT₁ and AT₂ receptors may exist.

Cloning studies have identified at least two subtypes of the AT₁ receptor which have been termed AT_{1A} and AT_{1B} (Murphy *et al.*, 1991; Iwai & Inagami, 1992). Moreover, Zhou *et al.* (1993) have identified two distinct binding sites in rat mesangial cells, coupled to G proteins, which are sensitive to both losartan and PD 123319. One binding site, which showed a much higher affinity for losartan was termed AT_{1A} by Zhou *et al.* (1993). The other binding site which showed a higher affinity for PD 123319 was termed AT_{1B} (Zhou *et al.*, 1993). The AT_{1A} and AT_{1B} subtypes are involved in the phospholipase C-mediated increase in intracellular calcium and inhibition of adenylyl cyclase evoked by angiotensin II in the rat mesangial cells (Madhun *et al.*, 1993). Only the AT_{1A} receptor is involved in the angiotensin II-induced increase in protein synthesis (Madhun *et al.*, 1993). Therefore, the original classification of AT₁ and AT₂ receptors may need to be extended to include AT_{1A} and AT_{1B} receptor subtypes.

In conclusion, the receptor involved in the enhancement of sympathetic neuroeffector transmission in the rat caudal artery and the rat vas deferens is blocked by both losartan and PD 123319. This suggests that the prejunctional receptor may be of the AT_{1B} subtype as described by Zhou *et al.* (1993). In contrast, the vasoconstrictor effect of angiotensin II in the rat caudal artery is only blocked by losartan and may be regarded as the AT_{1A} subtype.

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