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Multiple prejunctional actions of angiotensin II on noradrenergic transmission in the caudal artery of the rat

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1 Angiotensin II produced concentration-dependent enhancement of both stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline and stimulation-evoked vasoconstrictor responses in isolated preparations of rat caudal artery in which the noradrenergic transmitter stores had been labelled with [^{3}H]-noradrenaline. The threshold concentrations of angiotensin II for enhancement of S-I efflux (between 0.03 and 0.1 μM) and of the stimulation-evoked vasoconstrictor responses (about 0.3 μM) were 10-1000 times higher than those that have been found for several other vascular preparations.

2 The AT_1 angiotensin II receptor antagonist losartan (0.01 and 0.1 μM), reduced or abolished the enhancement of S-I efflux by 1 and 3 μM angiotensin II and the enhancement of vasoconstrictor responses by 1 μM angiotensin II. Surprisingly, the combination of 0.01 μM losartan and 0.1 μM angiotensin II enhanced S-I efflux to a much greater extent than did 0.1 μM angiotensin II alone. Moreover, the combination of 0.01 μM losartan and 0.1 μM angiotensin II enhanced stimulation-evoked vasoconstrictor responses, in contrast to the lack of effect of 0.1 μM angiotensin II alone.

3 In a concentration of 0.01 μM, the angiotensin II AT_2 receptor antagonist PD 123319 did not affect the enhancement of either S-I efflux or vasoconstrictor responses by angiotensin II. However, in a higher concentration (0.1 μM), PD 123319 antagonized the enhancement of both the S-I efflux and vasoconstrictor responses by angiotensin II.

4 In concentrations of 0.01 and 0.1 μM, PD 123319 prevented the marked enhancement of both S-I efflux and stimulation-evoked vasoconstrictor responses produced by the combination of 0.1 μM angiotensin II and 0.01 μM losartan.

5 The potentiation by losartan (0.01 μM) of the facilitatory effect of 0.1 μM angiotensin II on S-I efflux and on stimulation-evoked vasoconstriction was still observed in the presence of either the cyclo-oxygenase inhibitor indomethacin (3 μM), or the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM).

6 The findings confirm our previous suggestion that, in the rat caudal artery, angiotensin II receptors similar to the AT_{1B} subtype subserve enhancement of transmitter noradrenaline release.

7 The synergistic prejunctional interaction of 0.01 μM losartan and 0.1 μM angiotensin II may be due to either the unmasking by losartan of a latent population of angiotensin II receptors also subserving facilitation of transmitter noradrenaline release, or alternatively, losartan may block an inhibitory action of angiotensin II on transmitter noradrenaline release which normally opposes its facilitatory effect.

Keywords: Angiotensin II; angiotensin II receptors; losartan; PD 123319; rat caudal artery; noradrenergic transmission

Introduction

Angiotensin II, the primary effector hormone of the renin-angiotensin system, has a wide range of physiological actions directed at target organs in the cardiovascular system. These include vasoconstriction of peripheral blood vessels and facilitation of noradrenergic neuroeffector transmission, the latter being largely due to the ability of angiotensin II to enhance transmitter release from sympathetic nerves (Zimmerman, 1972; Starke, 1977; Story & Ziogas, 1987). The actions of angiotensin II throughout the body are subserved by specific receptors: two main angiotensin II receptor subtypes have been proposed on the basis of selective binding of non-peptide receptor antagonists (Chiu *et al.*, 1989; Whitebread *et al.*, 1989). Angiotensin II receptor sites sensitive to losartan have been termed AT_1, whereas those sensitive to PD 123319 have been designated AT_2. Nearly all of the known physiological actions of angiotensin II are blocked by losartan and are therefore presumed to be subserved by AT_1 receptors. Limited studies suggest an involvement of AT_2 receptors in several physiological actions of angiotensin II, but the role of AT_2 receptors remains uncertain. However, there is growing evidence that the two angiotensin II receptor subtypes cannot accommodate many recent observations, including findings from functional studies on the numerous actions of angiotensin II. On the basis of findings from studies which employed molecular biological techniques, further subdivision of AT_1 sites into AT_{1A} and AT_{1B} subtypes and of AT_2 sites into AT_{2A} and AT_{2B} subtypes, has been proposed (Ernsberger *et al.*, 1992; Madhun *et al.*, 1993; Zhou *et al.*, 1993). Furthermore, binding sites with a high affinity for PD 123319 and a somewhat lower affinity for losartan have been identified in several tissues and these are considered to be AT_{1B} sites (Madhun *et al.*, 1993; Zhou *et al.*, 1993).

In addition to the interactions of angiotensin II with noradrenergic neuroeffector transmission mentioned above, angiotensin II also liberates prostaglandins from cells in a number of tissues, including myocardial cells (Lanier & Malik, 1982), kidney (McGiff *et al.*, 1970) and vascular endothelial cells (Gimborne & Alexander, 1975). It has also been shown that angiotensin II releases prostaglandins from human astrocytes (Jaiswal *et al.*, 1991b) and rabbit vas deferens (Catalioto *et al.*, 1994) and that this action of the peptide is blocked by both AT_1 and AT_2 receptor antagonists. In blood vessels angiotensin II also induces the release of endothelium-derived smooth muscle relaxing factors such

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as prostacyclin and nitric oxide (Gruetter *et al.*, 1988; Boulanger *et al.*, 1995). These endothelium-derived products generally exert opposing influences to the facilitatory actions of angiotensin II on noradrenergic neuroeffector transmission (Malik, 1978; Ferrer *et al.*, 1992; Boulanger *et al.*, 1995).

Recently, we reported preliminary findings which indicated that angiotensin II exerted an action on transmitter noradrenaline release in the rat caudal artery which apparently opposed the well-known prejunctional facilitatory effect of the peptide (Cox *et al.*, 1995b). Specifically, a low concentration of the AT_1-selective antagonist losartan (0.01 μM), was found to increase markedly the ability of angiotensin II to enhance the field stimulation-evoked efflux of [^{3}H]-noradrenaline which had been incorporated into the noradrenergic transmitter stores of the artery. The present paper presents a full account of our studies undertaken to characterize the actions of angiotensin II on noradrenergic neuroeffector transmission in the rat caudal artery and to identify the receptor subtypes involved. We have also investigated the possible involvement of locally generated cyclo-oxygenase products and/or nitric oxide in the interactions of angiotensin II with the transmission process. The experiments described were performed with isolated preparations of the caudal artery of rats. The noradrenergic transmitter stores of the arteries being labelled with [^{3}H]-noradrenaline. Measurements were made of the efflux of [^{3}H]-noradrenaline and the accompanying vasoconstrictor response evoked by stimulation of the adventitial sympathetic nerves of the artery preparations.

Methods

Rat caudal artery preparation

Sprague-Dawley rats (250–350 g) of either sex were killed by a blow to the head and exsanguinated. A length 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% O_2 and 5% CO_2 and maintained at 37°C. Artery preparations (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 preparations were perfused and superfused at a constant flow of 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) and recorded on a Macintosh IIci computer by use of a MacLab/4 data acquisition system and Chart v3.2.6. software (Analogue Digital Instruments, Sydney, Australia). The A/D sampling rate was between 250–1000 Hz. Circular, bipolar, platinum electrodes were placed around the preparations to enable periarterial sympathetic nerve stimulation. Artery preparations were allowed to equilibrate for 15 min before commencement of experimental procedures.

After the 15 min equilibration period, the adventitial sympathetic nerves were stimulated (5 Hz, 10 s) to assess their responsiveness and the suitability of the preparation for use in the study. Preparations which responded satisfactorily with vasoconstriction to the test period of stimulation were removed from the organ baths and incubated for 30 min in 2 ml of PSS containing [^{3}H]-noradrenaline (58.1 Ci mmol^{-1}, 0.1 μM). After incubation with [^{3}H]-noradrenaline, artery preparations were again set up and perfused with PSS as described above. After 60 min a 30 s train of 1 ms square wave pulses at a frequency of 5 Hz was applied to the preparations to facilitate removal of non-specifically bound radioactivity from the tissues. After a further 30 min of perfusion/superfusion, the artery preparations were subjected to two periods of electrical field stimulation (5 Hz, 30 s), delivered 30 min apart. For each period of stimulation, 6 consecutive 1-min collections (4 ml volume) of the perfusate/superfusate were taken for measurement of the efflux of [^{3}H]-noradrenaline from the artery preparations, the train of stimulation being given after the second collection. The perfusate/superfusate was collected in ice-cold glass vials containing 10 μl Na_2SO_3 (1 M) 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 [^{3}H]-noradrenaline from its tritiated metabolites.

In experiments in which the effects of angiotensin II and/or non-peptide angiotensin II antagonists were investigated, these drugs were introduced into the PSS 20 min before the second period of stimulation.

In experiments in which the effects of indomethacin and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were investigated, these drugs were introduced into the PSS 30 min before the first period of stimulation and were present for the remainder of the experiment.

Separation of [^{3}H]-noradrenaline from its metabolites

[^{3}H]-noradrenaline was separated from its metabolites by column chromatography with alumina and Dowex-50W ion-exchange resin, by 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 passed through an alumina column which adsorbed the catechol compounds noradrenaline and 3,4-dihydroxyphenylethylene-glycol (DOPEG). Noradrenaline and DOPEG were then eluted from the alumina columns with two 1 ml aliquots of acetic acid (0.4 M). Noradrenaline was separated from DOPEG by adsorption on Dowex-50W columns, the noradrenaline being eluted with two 0.5 ml aliquots of 6 M HCl/ethanol (1 : 1, v/v). To determine the recovery of [^{3}H]-noradrenaline from the columns, a known quantity of purified [^{3}H]-noradrenaline was routinely passed through the above procedures. The values of resting and stimulation-induced (S-I) effluxes given have not been corrected for recovery ($78.0 \pm 1.8\%$, $n = 16$).

Determination of [^{3}H]-noradrenaline effluxes and vasoconstrictor responses

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 automatic external standardisation and the results expressed as disintegrations per minute (d.p.m.).

The resting efflux of [^{3}H]-noradrenaline preceding each of the two periods of stimulation (R_1 and R_2) was determined as the mean content of [^{3}H]-noradrenaline in the two 1-min collections of the perfusate/superfusate solution taken immediately before each period of stimulation. The S-I efflux of [^{3}H]-noradrenaline for each stimulation period was calculated by subtracting the corresponding resting efflux from the content of [^{3}H]-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 [^{3}H]-noradrenaline for the second period of stimulation were each expressed as a percentage of their corresponding value for the first period of stimulation, (% R_2/R_1) and (% S_2/S_1), respectively.

The stimulation-evoked vasoconstrictor responses were measured as increases in perfusion pressure (mmHg). For each preparation the response to the second period of stimulation was expressed as a percentage of that to the first period of stimulation (% V_2/V_1).

Drugs and radiochemicals

The physiological salt solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03 and D-(+)-glucose 11.1. Ethylenediaminetetraacetic acid disodium salt (0.067 mM) and ascorbic acid (0.14 mM) 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), (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 (PD 123319) (gift from Parke Davis, Ann Arbor, Michigan), indomethacin (Merck Sharp and Dohme, Australia) and N^{ω}-nitro-L-arginine methyl ester (Sigma, U.S.A.).

Angiotensin II stock solutions were prepared in concentrations of 1 mM in 5% acetic acid and stored in 50 μl aliquots at 0°C. Stock solutions of losartan and PD 123319 (1 mM) were prepared in deionised water and stored at 5°C. A stock solution of indomethacin (1 μM) was prepared in 0.01 μM Na_2CO_3 and stored at 5°C. L-NAME was prepared freshly each day in deionised water to a concentration of 1 mM. All stock solutions were diluted in PSS and added to the reservoirs supplying the organ bath.

Tritiated noradrenaline ([2,5,6-^{3}H]-(−)-noradrenaline) was suppled by the Radiochemical Centre, Amersham, U.K., with a specific activity of 58.1 Ci mmol^{-1} and a radioactive concentration of 1 mCi ml^{-1}.

Statistical analysis

Data are expressed as means ± standard error of the mean (s.e.mean); *n* denotes the number of experiments. The statistical significance of differences between means was determined by unpaired, two-tailed Student's *t* test or, in the case of multiple (more than two) groups, by first testing for global differences between experimental means by analysis of variance (ANOVA). Student-Newman-Keuls (SNK) test was used to test for significant differences between predetermined pairs of means. The significance of differences between the effects of individual concentrations of angiotensin II and the relevant control value was determined by Dunnett's test. All statistical analyses were performed with the computer programme Sigmastat for Windows (Version 1.0, Jandel Scientific). In all cases probability levels less than 0.05 ($P < 0.05$) were taken to indicate significant differences.

Results

Control experiments

Rat caudal artery preparations loaded with [^{3}H]-noradrenaline were subjected to two periods of electrical field stimulation (5 Hz, 30 s), delivered 30 min apart. In eight control experiments, the absolute mean resting efflux of [^{3}H]-noradrenaline, preceding the first period of stimulation (R_1), was 201.0 ± 18.7 d.p.m. per 1-min collection. There was a decline in the resting efflux of [^{3}H]-noradrenaline between the two periods of stimulation, such that the resting efflux preceding the second period of stimulation, expressed as a percentage of that preceding the first (% R_2/R_1), had a mean value of 82.9 ± 5.1%. Stimulation of the periarterial sympathetic nerves produced an increased efflux of [^{3}H]-noradrenaline. The S-I efflux of [^{3}H]-noradrenaline evoked by the first period of stimulation was 725.5 ± 144.7 d.p.m. The S-I efflux of [^{3}H]-noradrenaline with the second period of stimulation, expressed as a percentage of that for the first (% S_2/S_1), had a mean value of 96.6 ± 6.6%.

Field stimulation of the caudal artery preparations also produced vasoconstriction. In the eight control experiments, the mean increase in perfusion pressure with the first of the two periods of stimulation was 98.1 ± 26.1 mmHg. The vasoconstrictor response with the second period of stimulation, expressed as a percentage of that with the first (% V_2/V_1), had a mean value of 98.2 ± 4.1%.

Effects of angiotensin II on S-I efflux and stimulation-evoked vasoconstrictor responses

Introduction of angiotensin II (0.01 − 3 μM) into the PSS, 20 min before the second period of stimulation, did not sig-

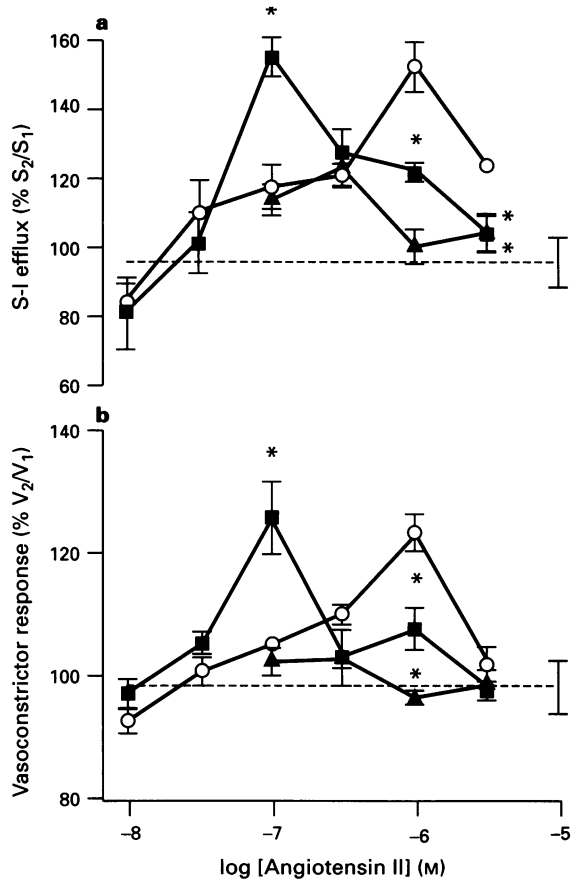

Figure 1 Effects of angiotensin II, in the absence and presence of losartan, on the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline (a) from rat caudal artery preparations and on the associated stimulation-evoked vasoconstrictor responses (b). The noradrenergic transmitter stores of the artery preparations had been radiolabelled with [^{3}H]-noradrenaline. The periarterial sympathetic nerves were stimulated for two 30 s periods (5 Hz) at 30 min intervals. In each experiment, the S-I efflux and vasoconstrictor response with the second period of stimulation were expressed as percentages of their corresponding values in the first period (% S_2/S_1 and % V_2/V_1, respectively). Angiotensin II (0.01 − 3 μM, ○) or combination of angiotensin II and losartan (0.01, ■ or 0.1 μM, ▲), were introduced into the PSS perfusing and superfusing the artery preparations 20 min before the second period of stimulation. The points plotted represent the means and vertical lines the s.e.mean from 4 − 9 experiments (note that in some cases these were smaller than the symbol representing the mean). The broken horizontal lines in each panel represent the mean values of % S_2/S_1 and % V_2/V_1 obtained in the absence of drugs, the s.e.means being shown at the right hand end of the lines. Note that the data for losartan alone are given in the text. Angiotensin II significantly enhanced S-I efflux (ANOVA), there being a significant difference from control with 0.1, 0.3, 1 and 3 μM angiotensin II (Dunnett's test). The effect of angiotensin II on the vasoconstrictor response to stimulation was also significant (ANOVA), the mean responses in the presence of 0.3 and 1 μM angiotensin II differing significantly from control (Dunnett's test). The asterisks indicate that the mean S-I efflux or stimulation-evoked vasoconstrictor response in the presence of a combination of losartan and angiotensin II was significantly different from that of angiotensin II alone (SNK test).

nificantly alter the resting efflux of [^{3}H]-noradrenaline (data not shown, ANOVA-SNK). However, as shown in Figure 1a, angiotensin II produced a concentration-dependent increase in the S-I efflux of [^{3}H]-noradrenaline. The threshold concentration of angiotensin II was between 0.03 and 0.1 μM and the maximum enhancement of S-I efflux was produced by 1 μM angiotensin II. With a higher concentration (3 μM), the enhancement was less (Figure 1a).

Angiotensin II also produced concentration-dependent enhancement of the stimulation-evoked vasoconstrictor responses. The maximum enhancement of stimulation-evoked vasoconstriction was produced by 1 μM angiotensin II and a higher concentration of 3 μM was without significant effect on stimulation-evoked vasoconstriction (Figure 1b).

Effects of losartan on the enhancement of S-I efflux and vasoconstriction by angiotensin II

Losartan in concentrations of 0.01 and 0.1 μM, added to the PSS 20 min before the second period of stimulation, did not significantly alter either the resting or the S-I efflux of [^{3}H]-noradrenaline (t tests). The mean values of % R_2/R_1 in the presence of 0.01 μM and 0.1 μM losartan were 83.0±7.2% (n=4) and 75.0±4.4% (n=4), respectively. The mean values of % S_2/S_1 in the presence of 0.01 and 0.1 μM losartan were 103.5±6.9% and 108.1±7.5%, respectively.

The effect of losartan on the enhancement of S-I efflux by angiotensin II was dependent upon the concentrations of both losartan and angiotensin II. As shown in Figure 1a, the enhancing effect of angiotensin II in the two highest concentrations tested (1 and 3 μM), was reduced or abolished by both 0.01 and 0.1 μM losartan. The enhancement produced by 0.1 μM angiotensin II, was not altered by 0.1 μM losartan but 0.01 μM losartan markedly increased the enhancement of S-I efflux produced by 0.1 μM angiotensin II (Figure 1a). As for the peptide alone the combination of 0.01 or 0.03 μM angiotensin II with 0.01 μM losartan did not alter the S-I efflux.

Losartan alone, in concentrations of 0.01 or 0.1 μM, was also without significant effect (t tests) on the stimulation-evoked vasoconstrictor responses of the artery preparations, the values of % V_2/V_1, in the presence of 0.01 and 0.1 μM losartan being 101.4±1.2% (n=4) and 102.6±0.8% (n=4), respectively.

The effect of losartan on the enhancement of stimulation-evoked vasoconstrictor responses by angiotensin II paralleled the findings for S-I [^{3}H]-noradrenaline efflux. Thus, as shown in Figure 1b, the enhancement of % V_2/V_1 by 1 μM angiotensin II was reduced or abolished by losartan (0.01 and 0.1 μM). As with the peptide alone, in the presence of 0.1 μM losartan, 0.1 μM angiotensin II was without effect on the vasoconstrictor response to stimulation but, the combination of the lower concentration of losartan (0.01 μM) and 0.1 μM angiotensin II markedly enhanced the vasoconstrictor response to stimulation.

Effects of PD 123319 on the enhancement of S-I efflux and vasoconstriction by angiotensin II

PD 123319 in concentrations of 0.01 and 0.1 μM, added to the PSS 20 min before the second period of stimulation, did not significantly alter the resting or S-I efflux of [^{3}H]-noradrenaline (t tests). The mean values of % R_2/R_1 in the presence of 0.01 and 0.1 μM PD 123319 were 88.7±6.2% and 83.3±4.5% (n=4), respectively. The mean values of % S_2/S_1 in the presence of 0.01 and 0.1 μM PD 123319 were 100.7±7.7% and 108.0±4.1%, respectively.

The lower concentration of PD 123319 (0.01 μM), was without effect on the enhancement of S-I efflux by angiotensin II throughout the concentration range of angiotensin II with which it was tested (Figure 2a). In a ten fold higher concentration (0.1 μM), PD 123319 effectively shifted the concentration-response relationship for angiotensin II to the right, reducing or abolishing the enhancement by 0.1, 0.3 and 1 μM angiotensin II (Figure 2a). In contrast to the finding with losartan (0.01 μM), neither concentration of PD 123319 increased the effect of the lowest concentration of angiotensin II (0.1 μM) on S-I efflux.

PD 123319 in concentrations of 0.01 and 0.1 μM, when present alone, did not significantly alter the stimulation-evoked vasoconstrictor responses of the artery preparations (t tests), the values of % V_2/V_1 being 99.6±0.9% (n=4) and 97.4±2.9% (n=4).

The effect of combinations of PD 123319 and angiotensin II on the stimulation-evoked vasoconstrictor response paralleled the findings for S-I efflux. Thus, as shown in Figure 2b, the enhancements of the stimulation-evoked vasoconstrictor responses produced by 0.3 and 1 μM angiotensin II were not

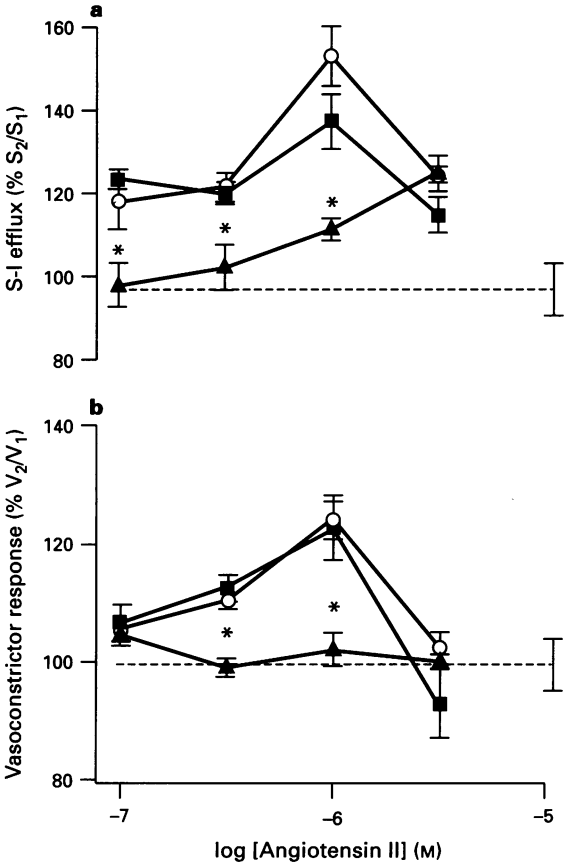

Figure 2 Effects of angiotensin II, in the absence and presence of PD 123319, on the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline (a) from rat caudal artery preparations and on the associated stimulation-evoked vasoconstrictor responses (b). The noradrenergic transmitter stores of the artery preparations had been radiolabelled with [^{3}H]-noradrenaline. The periarterial sympathetic nerves were stimulated for two 30 s periods (5 Hz) at 30 min intervals. In each experiment, the S-I efflux and vasoconstrictor response with the second period of stimulation were expressed as percentages of their corresponding values in the first period (% S_2/S_1 and % V_2/V_1, respectively). Angiotensin II (0.1–3 μM, ○) or combination of angiotensin II and PD 123319 (0.01 μM, ■ or 0.1 μM, ▲), were introduced into the PSS perfusing and superfusing the artery preparations 20 min before the second period of stimulation. The points plotted represent the means and vertical lines the s.e.mean from 4–8 experiments. The broken horizontal lines in each panel represent the mean values of % S_2/S_1 and % V_2/V_1 obtained in the absence of drugs, the s.e.means being shown at the right hand end of the lines. Note that the data for PD 123319 alone are given in the text. The asterisks indicate that the mean S-I efflux or stimulation-evoked vasoconstrictor response in the presence of a combination of PD 123319 and angiotensin II was significantly different from that of angiotensin II alone (SNK test).

altered by the lower concentration of PD 123319 (0.01 μM). In the higher concentration (0.1 μM), PD 123319 abolished the enhancement of stimulation-evoked vasoconstriction by 0.3 and 1 μM angiotensin II.

Effect of indomethacin on the interaction of angiotensin II and losartan on S-I efflux

The possibility was considered that the action of losartan in increasing the enhancement of S-I efflux by 0.1 μM angiotensin II, might be due to interference with angiotensin II-induced formation of a prostanoid with an inhibitory action on transmitter noradrenaline release. This was tested with the cyclo-oxygenase inhibitor indomethacin. Indomethacin (3 μM), when added alone to the PSS 30 min before the first period of stimulation, did not alter either the absolute resting or S-I efflux of [^{3}H]-noradrenaline with the first period of stimulation (data not shown, *t* tests). As shown in Figure 3, indomethacin was also without effect on the S-I efflux of [^{3}H]-noradrenaline with the second period of stimulation (% S_2/S_1).

In contrast to its effect alone, in the presence throughout of indomethacin, angiotensin II (0.1 μM), introduced for the second period of stimulation, did not enhance the S-I efflux of [^{3}H]-noradrenaline from artery preparations, rather it produced a small decrease of the evoked efflux (Figure 3). However, when 0.01 μM losartan was introduced together with angiotensin II (0.1 μM), S-I efflux was enhanced, despite the presence of indomethacin (Figure 3).

Indomethacin alone (3 μM), was also without significant effect (SNK test) on the stimulation-evoked vasoconstrictor responses of the artery preparations, the value of % V_2/V_1 in the presence of indomethacin being 99.8±1.6% ($n=7$). As in its absence, in the presence of indomethacin, angiotensin II in a concentration of 0.1 μM, did not significantly alter (SNK test) stimulation-evoked vasoconstriction (% $V_2/V_1=99.7\pm3.1\%$, $n=5$). Moreover, in the presence of indomethacin, stimulation-evoked vasoconstriction was again significantly enhanced (SNK test) when the lower concentration of losartan (0.01 μM) was introduced together with 0.1 μM angiotensin II (% $V_2/V_1=123.4\pm4.0\%$, $n=7$).

Effect of L-NAME on the interaction of angiotensin II and losartan on S-I efflux

Another possibility considered was that the increase in the enhancing action of 0.1 μM angiotensin II on S-I efflux by losartan (0.01 μM), might involve angiotensin II-induced nitric oxide production and an inhibitory action of nitric oxide on transmitter noradrenaline release. This was explored by use of the inhibitor of nitric oxide synthase, L-NAME. L-NAME (100 μM), added alone to the PSS 30 min before the first period of stimulation, was without effect on either the absolute resting or S-I efflux with the first period of stimulation (data not shown, *t* tests). As shown in Figure 4, L-NAME also did not alter the S-I efflux of [^{3}H]-noradrenaline with the second period of stimulation (% S_2/S_1).

In the presence throughout of L-NAME, 0.1 μM angiotensin II, introduced for the second period of stimulation, was without effect on the S-I efflux of [^{3}H]-noradrenaline (Figure 4). However, when in the presence of L-NAME, 0.01 μM losartan was introduced with angiotensin II (0.1 μM), S-I efflux was markedly enhanced (Figure 4).

L-NAME (100 μM), when present alone, was also without significant effect (SNK test) on the stimulation-evoked vasoconstrictor responses of the artery preparations, the value of % V_2/V_1 in the presence of L-NAME being 97.0±2.8% ($n=4$). As in its absence, in the presence of L-NAME, angiotensin II, in a concentration of 0.1 μM did not significantly alter stimulation-evoked vasoconstriction (% $V_2/V_1=105.5\pm5.3\%$, $n=4$) (SNK test). However, stimulation-evoked vasoconstric-

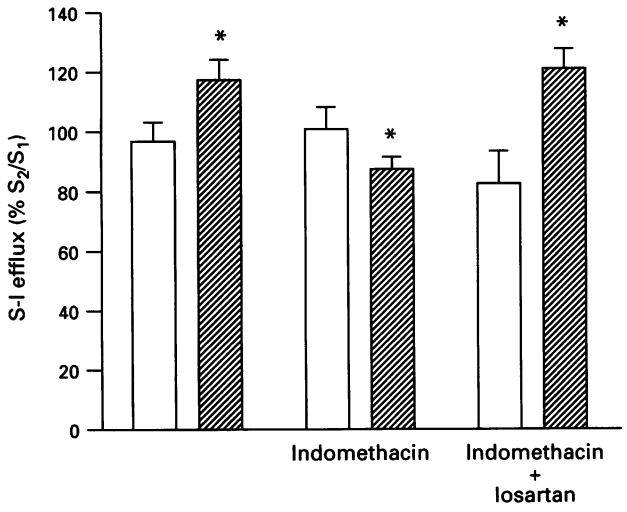

Figure 3 Effect of angiotensin II alone, in the presence of indomethacin and in the presence of indomethacin plus losartan on the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline from rat caudal artery preparations in which the noradrenergic transmitter stores had been labelled with [^{3}H]-noradrenaline. The periarterial sympathetic nerves were stimulated for two 30 s periods (5 Hz) at 30 min invervals. In each experiment, the S-I efflux with the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1). Indomethacin (3 μM) was introduced into the PSS 30 min before the first period of stimulation and then remained present throughout. Angiotensin II (0.1 μM, hatched column) and losartan (0.01 μM) were introduced into the PSS perfusing and superfusing the artery preparations 20 min before the second period of stimulation. The columns represent the means and the vertical lines the s.e.means from 4–9 experiments. The asterisks indicate significant differences between pairs of means (SNK test).

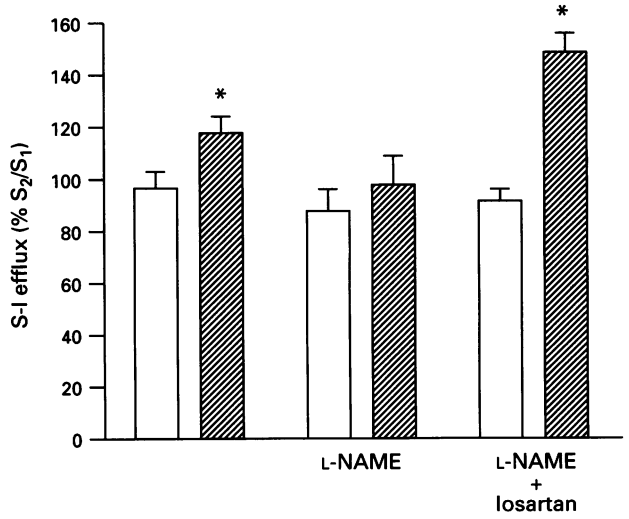

Figure 4 Effect of angiotensin II alone, in the presence of L-NAME and in the presence of L-NAME plus losartan on the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline from rat caudal artery preparations in which the noradrenergic transmitter stores had been labelled with [^{3}H]-noradrenaline. The periarterial sympathetic nerves were stimulated for two 30 s periods (5 Hz) 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 (% S_2/S_1). L-NAME (100 μM) was introduced into the PSS 30 min before the first period of stimulation and then remained present throughout. Where indicated, angiotensin II (0.1 μM, hatched columns) and losartan (0.01 μM) was introduced into the PSS perfusing and superfusing the artery preparations 20 min before the second period of stimulation. The bars represent the means and the vertical lines the s.e.means from 4–9 experiments. The asterisks indicate significant differences between pairs of means (SNK test).

tion was significantly enhanced when the lower concentration of losartan (0.01 μM) was introduced together with 0.1 μM angiotensin II, despite the presence of L-NAME (% $V_2/V_1 = 119.1 \pm 3.9\%$, $n = 5$), (SNK test).

Effect of PD 123319 on the interaction of angiotensin II and losartan on S-I efflux and vasoconstrictor responses

When 0.1 μM angiotensin II was introduced into the PSS in combination with 0.01 μM losartan plus either 0.01 or 0.1 μM PD 123319, the S-I efflux with the second period of stimulation did not differ from that obtained in the absence of angiotensin II, that is, with the two antagonists alone (Figure 5a). This was in contrast to the enhancement of S-I eflux produced by angiotensin II alone and the even greater enhancement produced by 0.1 μM angiotensin II in the presence of 0.01 μM losartan (Figure 5a).

PD 123319 (0.01 or 0.1 μM) also prevented the enhancement of stimulation-evoked vasoconstriction produced by the combination of 0.1 μM angiotensin II and 0.01 μM losartan (Figure 5b).

Discussion

It is generally accepted that the major action of angiotensin II on noradrenergic transmission is mediated prejunctionally, resulting in enhancement of transmitter release (Zimmerman, 1972; Starke, 1977; Story & Ziogas, 1987). However, in blood vessels, an action of angiotensin II on the smooth muscle cells, which increases their responsiveness to vasoconstrictor agents, contributes to the overall effect of the peptide on noradrenergic transmission (Zimmerman, 1972; Story & Ziogas, 1987). Findings from several studies indicate that the angiotensin AT_1 receptors are involved in the enhancement of sympathetic transmission by angiotensin II. Thus, the AT_1 receptor antagonist losartan but not the AT_2 receptor antagonist PD 123177 blocks the enhancement of vasoconstrictor responses to renal sympathetic stimulation produced by angiotensin II in the dog (Wong *et al.*, 1991). Likewise, both the pressor responses to spinal cord stimulation in pithed rats (Wong *et al.*, 1992) and S-I release of [^{3}H]-noradrenaline in brown adipose tissue (Cassis & Dwoskin, 1991) are selectively blocked by losartan. However, we have found that the enhancement of stimulation-induced efflux of [^{3}H]-noradrenaline produced by 1 μM angiotensin II in the caudal artery of Sprague-Dawley rats was blocked by 0.01 μM losartan and also by a 10 fold higher concentration of PD 123319 (Cox *et al.*, 1995a). The results of the present study confirm and extend these findings. Thus, the enhancements of stimulation-induced [^{3}H]-noradrenaline efflux produced by 1 and 3 μM angiotensin II in the rat caudal artery were markedly reduced or abolished by 0.01 μM losartan. PD 123319 in a 10 fold higher concentration (0.1 μM), also reduced the enhancement of S-I efflux produced by 1 μM angiotensin II, but not that produced by 3 μM angiotensin II. A lower concentration of PD 123319 (0.01 μM) was without effect on the enhancement of S-I efflux throughout the concentration range of angiotensin II with which it was tested. Similarly, the enhancement of the stimulation-evoked vasoconstrictor responses by 1 μM angiotensin II was reduced by 0.01 μM and abolished by 0.1 μM losartan, whereas only in the higher concentration (0.1 μM) did PD 123319 diminish the enhancement of the responses by angiotensin II (0.3 and 1 μM). It appears that both losartan and PD 123319 have affinity for the prejunctional angiotensin II receptor involved in the facilitatory action of the peptide, with losartan being about ten times more potent. We have also observed that the enhancement of both S-I efflux and stimulation-evoked vasoconstrictor responses produced in the rat caudal artery by 1 μM angiotensin II, is abolished by another AT_2 antagonist CGP 42112, in a concentration of 3 nM (unpublished findings). We previously suggested that the angiotensin II receptor subtype subserving enhancement of

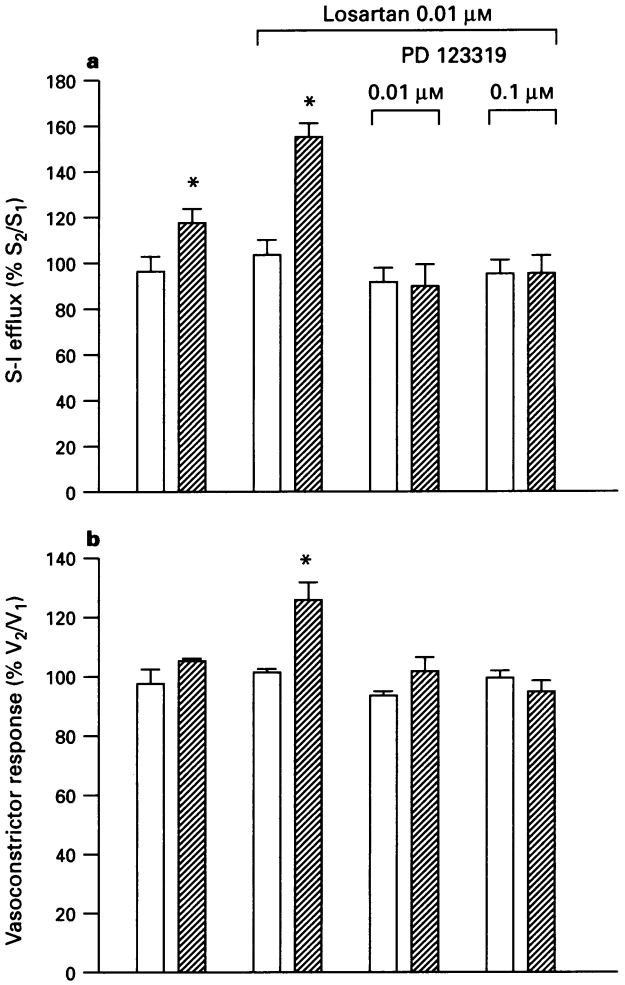

Figure 5 Effect of angiotensin II alone, in the presence of losartan and in the presence of losartan plus PD 123319 on the stimulation-induced (S-I) efflux of [^{3}H]-noradrenaline (a) from rat caudal artery preparations and on the associated stimulation-evoked vasoconstrictor response (b). The periarterial sympathetic nerves were stimulated for two 30 s periods (5 Hz) at 30 min intervals. In each experiment, the S-I efflux and vasoconstriction with the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1 and % V_2/V_1, respectively). Angiotensin II (0.1 μM, hatched columns) was introduced into the PSS perfusing and superfusing the artery preparations alone, or together with losartan (0.01 μM), or losartan plus PD 123319 (0.01 or 0.1 μM), 20 min before the second period of stimulation. The columns represent the means and the vertical lines the s.e.means from 4–9 experiments. The asterisks indicate significant differences between pairs of means (SNK test).

transmitter noradrenaline release in the rat caudal artery may be similar to the AT_{1B} subtype (Cox *et al.*, 1995a), as described by Zhou *et al.* (1993) and Madhun *et al.* (1993).

In the present study, angiotensin II in a lower concentration than those discussed above (0.1 μM), also enhanced the S-I efflux of [^{3}H]-noradrenaline, although the stimulation-evoked vasoconstrictor response was unaffected by angiotensin II in this concentration. Surprisingly, a combination of 0.1 μM angiotensin II and the lower concentration of losartan (0.01 μM) produced a much greater enhancement of S-I efflux than did angiotensin II alone. The combination also enhanced the stimulation-evoked vasoconstrictor response of the artery preparations, in contrast to the lack of effect of angiotensin II alone. The potentiation by losartan of the facilitatory effect of angiotensin II on noradrenergic transmission was dependent upon the concentration of both losartan and angiotensin II. Thus, the effects of 0.1 μM angiotensin II were not altered by a

higher concentration of losartan (0.1 μM) and the enhancements produced by higher concentrations of angiotensin II (0.3–3 μM) were not potentiated by losartan.

Jaiswal *et al.* (1991a) have described agonist-like actions of losartan in rat C6 glioma cells, human astrocytes and porcine aortic smooth muscle cells. In their studies, losartan stimulated the release of prostacyclin, both in the absence and the presence of angiotensin II. Mizuno *et al.* (1992) have shown that losartan can enhance local angiotensin II production in the vasculature of isolated hind legs of the rat. These findings suggest that under certain conditions, losartan may have angiotensin II agonist activity or act to promote local angiotensin II synthesis. However, other studies have produced contrary findings. Thus, losartan failed to stimulate the release of prostacyclin from porcine aortic smooth muscle cells, rat C6 glioma cells, rat spleen macrophages, porcine endothelial cells or bovine pulmonary arterial endothelial cells (Leung *et al.*, 1991). Furthermore, in human platelets, losartan failed to stimulate the release of endogenous thromboxane or lipoxygenase products (Liu *et al.*, 1992). Moreover, in the present study, losartan alone, did not alter the S-I efflux of [^{3}H]-noradrenaline or the vasoconstrictor responses of caudal artery preparations to sympathetic stimulation. Our findings suggest that losartan is not exerting angiotensin II agonist effects or inducing local angiotensin II production in the rat caudal artery, at least in so far as its influence on transmitter noradrenaline release or the evoked vasoconstriction are concerned.

Angiotensin II has been shown to stimulate the release of prostaglandins in the central nervous system and also in peripheral tissues (Nasjletti & Malik, 1982). The prostaglandins produced generally have an inhibitory influence on sympathetic neuroeffector transmission. Thus, the enhancement of transmitter noradrenaline release by angiotensin II is attenuated by prostaglandin production in the dog and rat heart (Lanier & Malik, 1982; 1983), guinea-pig vas deferens (Fredholm & Hedqvist, 1973) and cat spleen (Hedqvist *et al.*, 1971). Similarly, the vasoconstrictor response to angiotensin II in the rat renal artery (Aiken & Vane, 1973) is attenuated by prostaglandins. In the present study, blockade of prostaglandin synthesis by the cyclo-oxygenase inhibitor indomethacin, reduced the enhancement of S-I efflux produced by angiotensin II (0.1 μM). This finding is not consistent with the data mentioned above and suggest that, in the rat caudal artery, angiotensin II liberates a cyclo-oxygenase product(s) which facilitates transmitter noradrenaline release. However, despite the inhibition of S-I efflux of [^{3}H]-noradrenaline produced by the combination of indomethacin and angiotensin II, the combination of losartan (0.01 μM), angiotensin II (0.1 μM) and indomethacin (3 μM), enhanced S-I efflux to a greater extent than did angiotensin II alone (0.1 μM). This indicates that the potentiation by losartan of the angiotensin II enhancement of noradrenergic transmission cannot be due to the activation of prostanoid synthesis by angiotensin II.

There is increasing evidence that endothelium-derived nitric oxide (EDNO) plays a role in modulating the actions of angiotensin II (Chaki & Inagami, 1993; Kumagai *et al.*, 1993; Zhang *et al.*, 1994a). EDNO, like prostaglandins, generally oppose the actions of angiotensin II. Thus, in rabbit isolated aorta (Zhang *et al.*, 1994a), rat caudal artery (Thorin & Atkinson, 1994), renal artery (Zhang *et al.*, 1994b) and carotid artery (Boulanger *et al.*, 1995), inhibition of EDNO potentiates the vasoconstrictor actions of angiotensin II. Similarly, in two-kidney, one clip renovascular hypertension in the rat, EDNO synthesis increases in the non-clipped kidney, which counteracts the constrictor influence of elevated circulating angiotensin II (Sigmon & Beierwaltes, 1993). Administration of the EDNO synthase inhibitor L-NAME in the drinking water of rats, has been shown to increase blood pressure, as well as plasma renin activity (Ribeiro *et al.*, 1992). EDNO has also been shown to inhibit the dipsogenic effect of angiotensin II (Calapai *et al.*, 1992). Furthermore, the inhibitory action of angiotensin II on proximal tubular reabsorption in the rat is enhanced by EDNO inhibition. In the present study, the enhancement by angiotensin II of the S-I efflux of [^{3}H]-noradrenaline was reduced by L-NAME. Our data are at variance with those described above and suggest that EDNO may be exerting a facilitatory influence on sympathetic transmission in the rat caudal artery. Nevertheless, the increase in the enhancing effect of 0.1 μM angiotensin II on noradrenergic transmission produced by 0.01 μM losartan was still observed, despite the presence of L-NAME, suggesting that EDNO is not involved in the interaction of angiotensin II and losartan.

The log concentration-response relationships for both the enhancement of S-I [^{3}H]-noradrenaline efflux and the enhancement of vasoconstrictor responses by angiotensin II were shifted to the left by the lowest concentration of losartan (0.01 μM). Taken alone, these observations suggest that losartan has the ability to induce an increase in the affinity of the AT_1 receptor (putative AT_{1B}) subserving the prejunctional facilitatory action of angiotensin II on transmitter release. However, potentiation of the enhancing effect of the low concentration of angiotensin II on noradrenergic transmission by losartan was prevented by the AT_2 receptor antagonist PD 123319, in concentrations of both 0.01 and 0.1 μM. As discussed above, only in the higher concentration did PD 123319 prevent the enhancement of S-I efflux and the associated vasoconstrictor response by higher concentrations of angiotensin II in the absence of losartan. It is worthy of note that the AT_2 receptor antagonist CGP 42112 in a concentration of 3 nM, also prevented the synergistic interaction of 0.1 μM angiotensin II and 0.01 μM losartan on S-I efflux and stimulation-evoked vasoconstriction in the rat caudal artery (unpublished findings). A recent study indicates that PD 123319 in concentrations greater than 0.5 μM cross-reacts with the AT_{1B} receptor subtype (De Gasparo *et al.*, 1995). Based on this study, at least the lower concentration of PD 123319 (0.01 μM) could be considered to have a selective action on AT_2 receptors. This raises the possibility of an involvement of AT_2 subtype receptors in the interaction between losartan and 0.1 μM angiotensin II, rather than a losartan-induced increase in the affinity of the AT_1 receptor subserving facilitation of transmitter noradrenaline release.

The mechanism of the synergistic interaction between losartan and angiotensin II, in respect of the enhancement of S-I [^{3}H]-noradrenaline efflux and stimulation-evoked vasoconstriction, may be similar to that suggested by Hong *et al.* (1994) to explain the unmasking of a contractile effect of normally subthreshold concentrations of angiotensin II by AT_2 antagonists in rabbit abdominal aortic preparations. Hong *et al.* (1994) showed that, in the presence of the AT_2 antagonists PD 121981 and CGP 42112, angiotensin II, in concentrations which were normally subthreshold, had a contractile effect which was antagonised by low concentrations of losartan. It was proposed that the AT_2 antagonists induced up-regulation of 'latent' AT_1 receptors on the vascular smooth muscle, thereby allowing angiotensin II to exert an additional contractile action in very low concentrations. In our study, in the rat caudal artery, losartan enhanced the prejunctional facilitatory action of 0.1 μM angiotensin II on noradrenergic transmission and the effect of the combination of losartan and angiotensin II was prevented by a low concentration of PD 123319 (0.01 μM). Thus, by analogy with the conclusion of Hong *et al.* (1994), losartan may be 'unmasking' a latent population of AT_2 receptors which, in addition to the proposed AT_{1B}-like receptors activated by higher concentrations of angiotensin II, subserve facilitation of transmitter release. One difficulty with this explanation is that there was no synergistic interaction between losartan in a higher concentration (0.1 μM) and angiotensin II. However, with this higher concentration of losartan it is possible that the consequences of 'unmasking' the putative facilitatory AT_2 receptors on transmitter release, may be offset by blockade of facilitatory AT_{1B} receptors.

Rather than receptor up-regulation, the interaction of 0.1 μM angiotensin II and the lower concentration of losartan on noradrenergic transmission in the caudal artery might be

explained by opposing dual actions of angiotensin II on transmitter release. Thus, the well-known prejunctional facilitatory action of angiotensin II might be offset by an inhibitory action. However, it is difficult to reconcile all of the available angiotensin II concentration-effect data, in the presence and absence of AT_1 and AT_2 antagonists, with such a proposal. Nevertheless, as previously discussed, in the rat caudal artery, the prejunctional facilitatory action of angiotensin II in the higher concentration range appears to be subserved by receptors similar to the AT_{1B} subtype. It could be argued that an opposing prejunctional inhibitory action of angiotensin II is subserved by receptors similar to the AT_{1A} subtype. Thus, in a low concentration, losartan, which has some selectivity for AT_{1A} over AT_{1B} sites (Ernsberger *et al.*, 1992; Zhou *et al.*, 1993), might enhance the action of 0.1 μM angiotensin II by blocking the putative AT_{1A}-like inhibitory receptors. The failure of a ten fold higher concentration of losartan to alter the effect of angiotensin II on S-I [^{3}H]-noradrenaline efflux or vasoconstrictor responses might be due to blockade of both the putative inhibitory receptors (AT_{1A}-like) as well as the facilitatory receptors (AT_{1B}-like). However, it is difficult to account for the ability of PD 123319 in the lower concentration (0.01 μM), in which it is presumably selective for AT_2 receptors, to prevent the synergistic interaction between losartan and angiotensin II.

It is worthy of note that the maximal effect of angiotensin II alone, on S-I efflux of [^{3}H]-noradrenaline from rat caudal artery preparations, was observed with a concentration of 1 μM. This is 10 to 1000 times the concentration of the peptide shown to produce maximal enhancement of noradrenergic transmission in other tissues, including rat mesenteric artery (Munday *et al.*, 1983), rat caudal artery (Story & Ziogas, 1986) and in guinea-pig (Brasch *et al.*, 1993) and rat atria (Gironacci *et al.*, 1994). The apparent low potency of angiotensin II in enhancing transmitter release in the caudal artery might be due to a prejunctional inhibitory action of angiotensin II which opposes its enhancing action on transmitter release.

In summary, the present study has revealed a synergistic interaction of the angiotensin II AT_1 receptor antagonist losartan and angiotensin II on transmitter noradrenaline release in the rat caudal artery. Whilst the mechanism of the interaction remains obscure, it would appear to warrant further study. This is reinforced by our subsequent observations with caudal artery preparations from spontaneously hypertensive rats of a similar synergistic interaction between PD 123319, rather than losartan, and the relatively low concentration of angiotensin II (0.1 μM) (Cox *et al.*, 1996).

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