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Different receptors for angiotensin II at pre- and postjunctional level of the canine mesenteric and pulmonary arteries

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1 This investigation was undertaken to compare pre- and postjunctional receptors involved in the responses of the canine mesenteric and pulmonary arteries to angiotensin II.

2 In the mesenteric artery, angiotensin II caused an enhancement of tritium overflow evoked by electrical stimulation ($EC_{30\%} = 5$ nM), the maximal effect representing an increase by about 45%. Postjunctionally, angiotensin II caused concentration-dependent contractions ($pD_2 = 8.57$). Saralasin antagonized both pre- and postjunctional effects of angiotensin II, but it was more potent at post- than at prejunctional level (pA_2 of 9.51 and 8.15, respectively), while losartan antagonized exclusively the postjunctional effects of angiotensin II ($pA_2 = 8.15$). PD123319 had no antagonist effect either pre- or postjunctionally.

3 In the pulmonary artery, angiotensin II also caused an enhancement of the electrically-evoked tritium overflow ($EC_{30\%} = 1.54$ nM), its maximal effect increasing tritium overflow by about 80%. Postjunctionally, angiotensin II caused contractile responses ($pD_2 = 8.52$). As in the mesenteric artery, saralasin antagonized angiotensin II effects at both pre- and postjunctional level and it was more potent postjunctionally (pA_2 of 9.58 and 8.10, respectively). Losartan antagonized only the postjunctional effects of angiotensin II ($pA_2 = 7.96$) and PD123319 was ineffective.

4 It is concluded that in both vessels: (1) pre- and postjunctional receptors belong to a different subtype, since they are differently antagonized by the same antagonists; (2) postjunctional receptors belong to AT_1 subtype, since they are blocked by losartan but not by AT_2 antagonists; (3) prejunctional receptors apparently belong to neither AT_1 or AT_2 subtype since they are blocked by neither AT_1 nor AT_2 antagonists.

Keywords: Canine mesenteric artery; canine pulmonary artery; angiotensin II receptors; prejunctional effects; postjunctional effects; saralasin; losartan; PD123319

Introduction

Angiotensin II, the main effector product of the renin-angiotensin system, is known to act on at least two pharmacologically distinct receptors: AT_1, which are selectively blocked by losartan (Dudley *et al*., 1990; Bumpus *et al*., 1991; Duncia *et al*., 1992) and AT_2, which are selectively blocked by the compound PD123319 (Chiu *et al*., 1989; Dudley *et al*., 1990; Blankley *et al*., 1991). The angiotensin II binding site antagonized by losartan (AT_1 receptors) was the predominant receptor found in the vascular smooth muscle, adrenal cortex, liver and some regions of the brain. The angiotensin II binding site antagonized by PD123319 (AT_2 receptors) was predominant in uterine smooth muscle, ovary, adrenal medulla, the developing rat foetus and some brain regions (Zhou *et al*., 1993). On the basis of data obtained in both cloning and receptor binding studies, further subdivision of AT_1 receptors into AT_{1A} and AT_{1B} subtypes (Murphy *et al*., 1991; Iwai & Inagami, 1992) and of AT_2 into AT_{2A} and AT_{2B} subtypes has been proposed (Cox *et al*., 1995).

Under a functional point of view, AT_1 receptors mediate all the effects until now known caused by angiotensin II at postjunctional level: vascular smooth muscle contraction (Chiu *et al*., 1991; Wong *et al*., 1991a), aldosterone release (Wong *et al*., 1990a) and the regulation of fluid electrolyte balance (Barbella *et al*., 1993). No functional role has been reported yet for AT_2 receptors.

At prejunctional level, angiotensin II facilitates noradrenaline release from sympathetic varicosities (Zimmerman & Whitmore, 1967; Starke, 1977) and this effect was proposed to be mediated by AT_1 receptors (Parrish & Cassis, 1991; Brasch *et al*., 1993). In preliminary studies carried out in the canine mesenteric artery and in the rat left ventricle it was observed that neither losartan nor the compound PD123319 changed the facilitatory effect exerted by angiotensin II on the overflow of noradrenaline evoked by electrical stimulation (Guimarães *et al*., 1997). The present study was undertaken to compare the receptors involved in pre- and postjunctional responses of the canine mesenteric and pulmonary arteries to angiotensin II.

Methods

In the municipal dog pound, mongrel dogs, 9–16 kg in weight, of either sex, were anaesthetized with pentobarbitone sodium (30 mg kg^{-1} i.v. injected in the forelimb). Immediately after removal, the mesenteric and pulmonary arteries were placed in adequate bottles containing aerated (95% O_2 and 5% CO_2) and cold modified Krebs-Henseleit solution (Guimarães & Osswald, 1969) of the following composition (mM): NaCl, 118.6; KCl, 4.70; $CaCl_2$, 2.52; KH_2PO_4, 1.18; $MgSO_4$, 1.23; $NaHCO_3$, 25.0; glucose, 10.0. To avoid spontaneous oxidation of ^{3}H-noradrenaline, EDTA 0.027 and ascorbic acid 0.57 mM were added to the medium. The animals were sacrificed by an overdose of pentobarbitone sodium (100 mg kg^{-1}). The arteries were then transported to the laboratory where they were cut into small strips (of about 1.5×20 mm) to study

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prejunctional effects or into small rings (of about 5 mm length) to study postjunctional effects.

Prejunctional effects

To study prejunctional effects, the strips were preincubated for 30 min in 3 ml medium containing 1 mM pargyline (to inhibit monoamine oxidase), 40 μM hydrocortisone and 40 μM U-0521 (3,4-dihydroxy-2-methylpropiophenone) to inhibit extraneuronal removal (Guimarães *et al.*, 1978). These drugs (except pargyline) were kept in the medium for the remainder of the experiment. After preincubation, the strips were exposed for 60 min to ^{3}H-noradrenaline (0.2 μM). Thereafter they were mounted in a perifusion chamber and perifused with amine-free medium (aerated and at 37°C) during 110 min at a flow rate of 0.8 ml min^{-1}. From $t = 110$ min ($t = 0$ min being the onset of the perifusion) the perifusion fluid was collected continuously in samples of 5 min. Three periods of transmural electrical stimulation (1 Hz, 2 ms, 100 V, during 5 min; Stimulator II X, Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied at min 120 (S_1), 160 (S_2), and 200 (S_3). The first period of electrical stimulation was disregarded, the second was taken as control and the third one was used to study the influence exerted by angiotensin II (in the absence or in the presence of antagonists).

The prejunctional effect of angiotensin II was determined by the increase of tritium overflow evoked by electrical stimulation. Angiotensin II was added to the perifusion fluid 20 min before S_3. When angiotensin II antagonists were used, their addition to the perifusion fluid was carried out 30 min before S_1. $EC_{30\%}$ values represent the molar concentration of angiotensin II that increased the evoked overflow by 30% and $pEC_{30\%}$ the negative logarithm of $EC_{30\%}$.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of loss min^{-1}). The fractional release per shock was calculated by dividing evoked tritium overflow by tritium present in the tissue at the beginning of the stimulation period and by the number of shocks. Drug effects are expressed as the ratio FR of tritium evoked by S_3 over that evoked by S_2. Each result was corrected for time-dependent changes as determined in parallel drug-free control experiments.

For the calculation of the overflow induced by electrical stimulation those 5 min samples were taken into account in which the overflow of tritium exceeded that in the last pre-stimulation control sample (usually this applied to the 3 or 4 samples collected during and after stimulation). The spontaneous outflow measured in the last pre-stimulation sample was assumed to represent the spontaneous outflow in subsequent samples; it was subtracted from the overflow determined in stimulation and post-stimulation samples. The 'total overflow of transmitter' was the sum of all increases (induced by a period of stimulation) above the spontaneous level of outflow of tritium.

Determination of tritium in the perifusate and in the tissue

After the experiment, the tissues were kept overnight in 2 ml of 0.2 M perchloric acid. Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta, LKB Wallac, Turku, Finland) in 2-ml aliquots of perifusate (or 0.5 ml of the acid extract of the tissue + 1.5 ml of Krebs-Henseleit solution), after addition of 8 ml of scintillation mixture (OptiPhase 'HiSafe' 3, LKB, Loughborough, Leics, England).

Postjunctional effects

From each segment, rings of about 5 mm length were obtained and mounted in a 10 ml bath containing aerated modified Krebs-Henseleit solution at 37°C. Two stainless steel wires (diameter 0.05 mm) were introduced into the lumen and then moved to stretch the vessel wall until a resting tension of 9.8–14.7 mN was reached. One of the wires was fixed to the bottom of the bath and the other to the isometric transducer. The mechanical responses were recorded on a Harvard Universal Oscillograph. The rings were allowed to stabilize for 2 h.

Concentration-response curves were obtained by single additions with half-log increments. After the response had reached the maximum the tissue was repeatedly washed out. Desensitization has been reported when repeated additions of angiotensin II were made; indeed, in preliminary experiments in the canine mesenteric and pulmonary arteries some desensitization was observed whenever the interval between two successive additions was less than 10 min. To avoid this phenomenon, the additions of angiotensin II to the medium were made with intervals of at least 45 min.

pD_2 values represent the negative logarithm of the molar concentration of the agonist that causes 50% of the maximal contraction and was calculated from the concentration-response curves by interpolation. pA_2 values were calculated according to the method of Van Rossum (1963) from the equation: $pA_2 = pA_x + \log (x-1)$ in which x represents the factor or the shift of the concentration-response curve to the right and pA_x the negative logarithm of the molar concentration of the antagonist which caused this shift.

Statistics

The results are expressed as arithmetic means $\pm$ s.e.m. One-way analysis of variance was used to test differences between unpaired results. A probability level of 0.05 or less was considered statistically significant.

Drugs

Angiotensin II acetate (Sigma, St. Louis, MO, USA); cocaine hydrochloride (Uquipa, Lisboa, Portugal); hydrocortisone 21-hemisuccinate sodium (Sigma); losartan (Merck Portuguesa, Lisboa, Portugal); ^{3}H-7-(−)-noradrenaline (18.2–21.1 Ci mmol^{-1}) (New England Nuclear, Dreieich, Germany); pargyline hydrochloride (Sigma); PD123319 ditrifluoroacetate (S(+)-1-(((4-(dimethylamino)-3-methylphenyl)methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol(4,5-c) pyridine-6-carboxylic acid ditrifluoroacetate) (Research Biochemicals Incorporated (RBI), Natick, USA); saralasin (Sar^1, Val^5, Ala^8)-Angiotensin II acetate (Sigma); U-0521 (3,4-dihydroxy-2-methylpropiophenone (Upjohn, Kalamazoo, MI, USA).

Results

Prejunctional effects of angiotensin II

In control experiments without agonists and antagonists the basal efflux of tritium decreased slowly with time. However, the fractional rate of loss remained constant with time ($1.31 \pm 0.17 \times 10^{-3}$ min^{-1}, $n = 12$, for the mesenteric and $5.60 \pm 0.57 \times 10^{-4}$ min^{-1}, $n = 10$, for the pulmonary artery). Furthermore, the overflow evoked by electrical stimulation remained also constant throughout the experiment, as shown by the ratio S_3/S_2 which is close to unity (0.98 ± 0.05 for the

mesenteric and 1.01 ± 0.06 for the pulmonary artery). The fractional release per shock was $1.29 \pm 0.19 \times 10^{-5}$ pulse^{-1}, $n = 12$, in the mesenteric and $1.73 \pm 0.17 \times 10^{-5}$ pulse^{-1}, $n = 10$, in the pulmonary artery.

Angiotensin II (2–100 nM) caused a concentration-dependent enhancement of tritium overflow evoked by electrical stimulation, the maximum effect representing an increase by $45.1 \pm 7.3\%$ ($n = 6$) in the mesenteric and $81.2 \pm 7.9\%$ ($n = 4$) in the pulmonary artery (Figures 1 and 2). The $pEC_{30\%}$ values for angiotensin II were 8.30 ± 0.38 ($n = 12$) and 8.81 ± 0.31 ($n = 12$) in the mesenteric and pulmonary arteries, respectively ($P = 0.31$, not significant).

The nonspecific angiotensin II receptor antagonist saralasin (up to 100 nM), which *per se* influenced neither the basal outflow of tritium nor tritium overflow evoked by electrical stimulation of both arteries, shifted the concentration-response curve for angiotensin II to the right ($pA_2 = 8.15 \pm 0.34$, $n = 5$, in the mesenteric and 8.10 ± 0.41, $n = 4$, in the pulmonary artery). In the concentrations usually reported as selective for AT_1 receptors (3–100 nM), losartan, which *per se* changed neither the basal nor the electrically evoked release of tritium in both the mesenteric and pulmonary arteries, had no effect on the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation in either artery. However, in a much higher concentration, losartan caused small shifts of the concentration-response curve for angiotensin II to the right in both arteries ($pA_2 = 5.24 \pm 0.18$, $n = 5$, in the mesenteric and 5.40 ± 0.21, $n = 4$, in the pulmonary artery) (Figures 1 and 2). The selective AT_2 receptor antagonist PD123319 (10–100 nM), which *per se* changed neither the basal nor the electrically evoked release of tritium in both the mesenteric and pulmonary arteries, also had no effect on the enhancement by angiotensin II of tritium overflow evoked by electrical stimulation in either artery (Figures 1 and 2).

Postjunctional effects of angiotensin II

At postjunctional level, angiotensin II (1–100 nM) caused concentration-dependent contractions, the maximum effect reaching 15.2 ± 1.6 mN mg^{-1} of tissue ($n = 12$) in the mesenteric and 1.23 ± 0.13 mN mg^{-1} of tissue ($n = 8$) in pulmonary artery. The pD_2 for angiotensin II was 8.57 ± 0.09 ($n = 14$), in the mesenteric and 8.52 ± 0.08 ($n = 10$), in the pulmonary artery. As shown in Figures 3 and 4, the nonspecific angiotensin II antagonist saralasin (2 and 4 nM), which did not change the tone of either preparation, caused marked displacements of the concentration-response curve for angiotensin II to the right ($pA_2 = 9.51 \pm 0.10$, $n = 6$, in the mesenteric

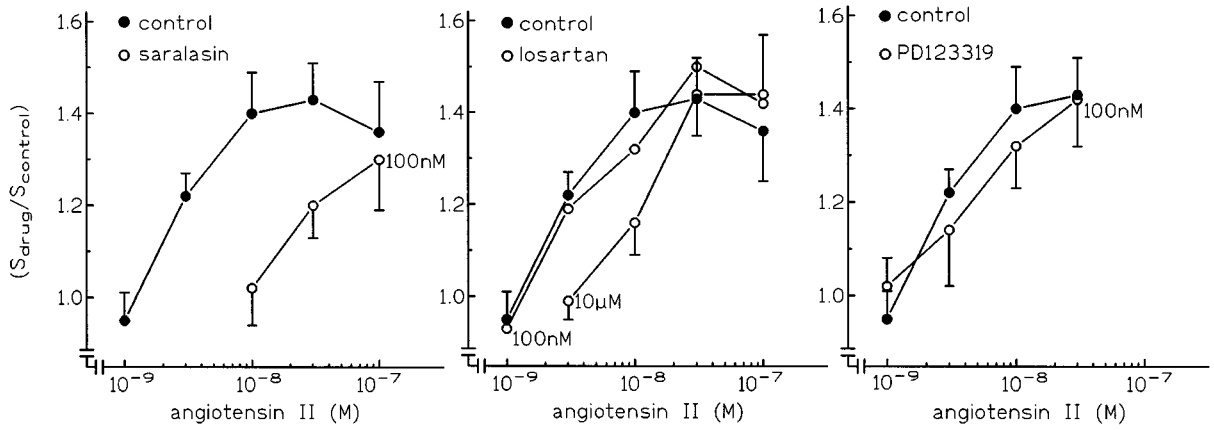

Figure 1 Canine mesenteric artery. Effects of saralasin, losartan and PD123319 on the enhancement by angiotensin II of the tritium overflow evoked by electrical stimulation (1 Hz, 2 ms, 100 V, during 5 min) before (control) and after saralasin (100 nM), losartan (100 nM or 10 μM) or PD123319 (100 nM). The results are means$\pm$s.e.m. of 5–6 experiments. For the sake of clarity, the bars indicating s.e.m. for 100 nM losartan were omitted.

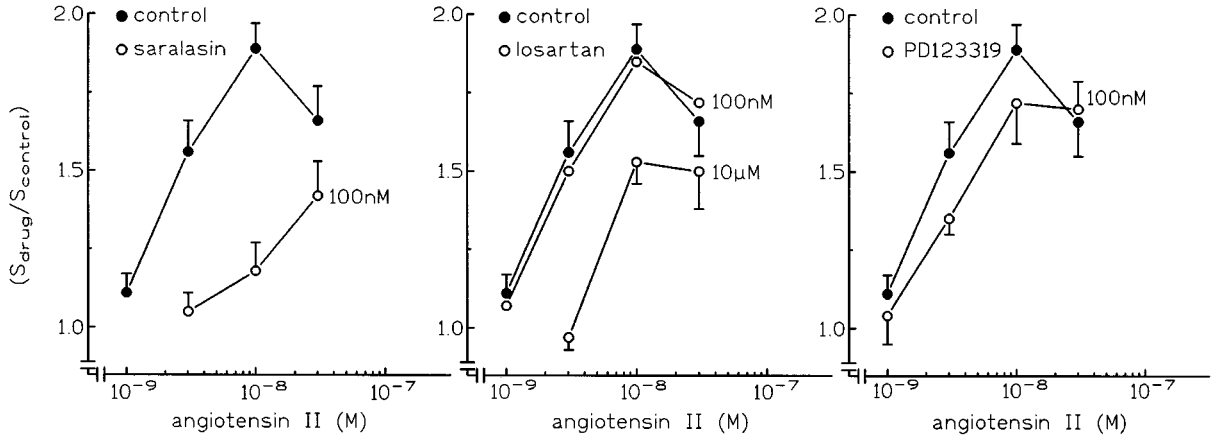

Figure 2 Canine pulmonary artery. Effects of saralasin, losartan and PD123319 on the enhancement by angiotensin II of the tritium overflow evoked by electrical stimulation (1 Hz, 2 ms, 100 V, during 5 min) before and after saralasin (100 nM), losartan (100 nM or 10 μM) or PD123319 (100 nM). The results are means$\pm$s.e.m. of 5–6 experiments. For the sake of clarity, the bars indicating s.e.m. for 100 nM losartan were omitted.

and 9.58 ± 0.12, $n = 6$, in the pulmonary artery). The selective AT_1 receptor antagonist losartan (25 and 100 nM), which *per se* also did not change the tone of the preparations, caused a concentration-dependent displacement of the concentration-response curve of angiotensin II to the right ($pA_2 = 8.15 \pm 0.12$, $n = 8$, in the mesenteric and 7.96 ± 0.14, $n = 6$, in the pulmonary artery; $P = 0.32$, not significant) (Figures 3 and 4). In contrast to saralasin and losartan, the selective AT_2 receptor antagonist PD123319 (up to 1 μM) had no effect on the response to angiotensin II in either vessel (Figures 3 and 4).

Discussion

The results reported in the present publication show that in the canine mesenteric and pulmonary arteries, either saralasin or losartan have different antagonist potencies at pre- and postjunctional level against angiotensin II. While saralasin was more potent against angiotensin II post- than prejunctionally (pA_2 values of 9.51 and 8.15, respectively, $P = 0.002$, in the mesenteric and 9.58 and 8.10, respectively, $P = 0.003$, in the pulmonary artery), losartan was a rather potent antagonist of angiotensin II postjunctionally (pA_2 of 8.15 in the mesenteric and of 7.96 in the pulmonary artery) and had no influence on angiotensin II effect prejunctionally, unless a very high concentration (10 μM), surely with non-specific antagonist effect, was used. This led to the conclusion that in the two canine arteries, prejunctional receptors which mediate the enhancement by angiotensin II of noradrenaline release evoked by electrical stimulation are different from postjunctional receptors mediating the contractions of the smooth muscle cells. The different potency of saralasin at the two levels and the lack of antagonist action of losartan at prejunctional level indicates the existence of different angiotensin II receptor subtypes pre- and postjunctionally.

Since losartan is widely recognized as a selective AT_1 receptor antagonist (Dudley *et al*., 1990; Trachte *et al*., 1991; Wong *et al*., 1991a), the postjunctional receptors of the canine mesenteric and pulmonary arteries which are blocked by losartan belong to AT_1 subtype, while those which mediate prejunctional effects of angiotensin II do not. This conclusion agrees with the general consensus that the postjunctional effects of angiotensin II are mediated by AT_1 receptors (Dudley *et al*., 1990; Wong *et al*., 1990b; Rhaleb *et al*., 1991; Cox *et al*., 1995). In contrast, there is no agreement regarding the subtype of receptors involved in prejunctional effects of angiotensin II. To which angiotensin II receptor subtype do prejunctional receptors of the canine mesenteric and pulmonary arteries belong? The question is not easy to answer, above all, because it has not yet been established how many

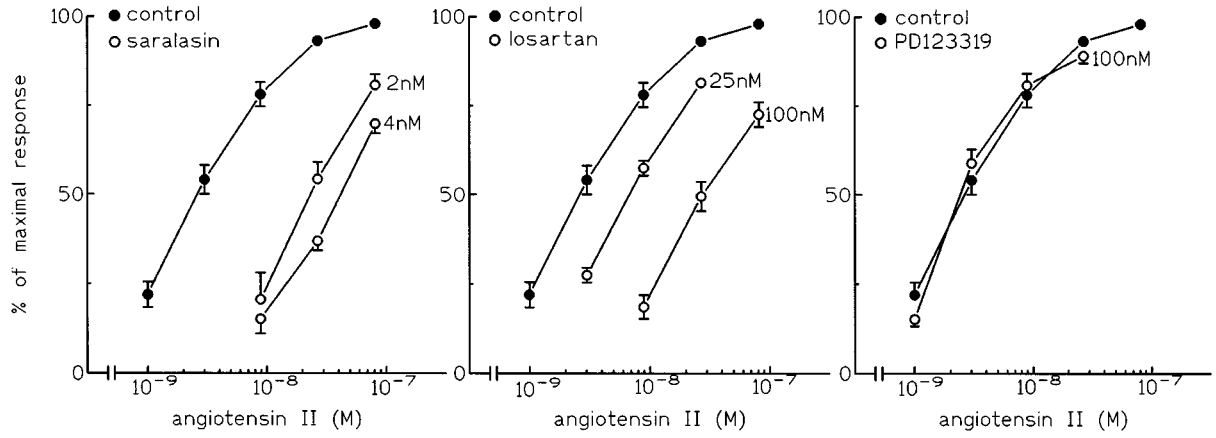

Figure 3 Canine mesenteric artery. Effects of saralasin, losartan and PD123319 on the concentration-dependent contractions to angiotensin II before and after saralasin (2 or 4 nM), losartan (25 or 100 nM) or PD123319 (100 nM). The results are means ± s.e.m. of 6–14 experiments.

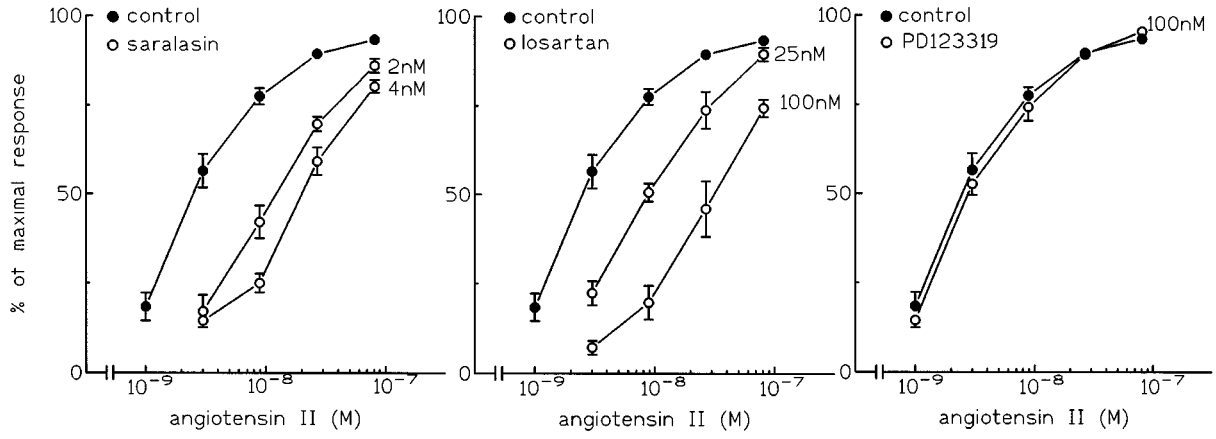

Figure 4 Canine pulmonary artery. Effects of saralasin, losartan and PD123319 on the concentration-dependent contractions to angiotensin II before and after saralasin (2 or 4 nM), losartan (25 or 100 nM) or PD123319 (100 nM). The results are means ± s.e.m. of 6–10 experiments.

angiotensin II receptor subtypes exist. In the majority of the tissues, prejunctional receptors for angiotensin II were classified as AT_1. In the isolated atria of the rat (Gironacci *et al*., 1994), in the isolated atria of guinea pig (Brasch *et al*., 1993), in the rabbit iris ciliary body (Ohio & Jumblatt, 1993) and in the rat tail artery (Cox *et al*., 1995) for example, the facilitatory effect of angiotensin II (0.1 – 3 μM) on noradrenaline release was inhibited by losartan (0.01 – 1 μM) but not by AT_2 receptor antagonists (PD 123319 or PD 123177: 0.01 – 0.1 μM). Similar inhibition by losartan of the facilitatory effect on noradrenaline release caused by angiotensin II was observed in the rat mesenteric vascular bed (Parrish & Cassis, 1991; Tofovic *et al*., 1991) and in the canine renal vascular bed (Wong *et al*., 1991b). Using the same or different tissues, other authors obtained different results. In the rat isolated tail artery, for example, Cox *et al*. (1995) found that the enhancement caused by angiotensin II of noradrenaline overflow evoked by electrical stimulation was antagonized by both the selective AT_1 receptor antagonist losartan and by the AT_2 selective receptor antagonist PD123319. Similar results were obtained in the rabbit vas deferens (Hedge & Clarke, 1993) and in the rat vas deferens (Cox *et al*., 1995). The comparison of these results with those obtained in the present study show that not only the pre- but also the postjunctional angiotensin II receptors of the canine mesenteric and pulmonary arteries are different from prejunctional angiotensin II receptors found in the tissues referred to above. In agreement with our results, both losartan (0.02 – 2 μM) and the AT_2 antagonist PD123177 failed to inhibit the enhancement of noradrenaline release by angiotensin II in the rabbit isolated vas deferens (Trachte *et al*., 1990; 1991) and these authors concluded that the enhancement of noradrenaline release evoked by electrical stimulation was neither AT_1 nor AT_2-receptors-mediated. Also *in vivo* (in pithed rats), sub-pressor doses of angiotensin II which shifted to the left the frequency-response curves for increases in blood pressure due to spinal cord stimulation, indicating a facilitatory effect on sympathetic transmitter outflow, were not antagonized by losartan (Ohlstein *et al*., 1997). Interestingly, the nonpeptide angiotensin II receptor antagonist eprosartan, which is chemically distinct from losartan, inhibited the enhancement by angiotensin II of pressor responses evoked by spinal cord stimulation (Ohlstein *et al*., 1997). Also in humans it was reported that stimulation of the sympathoadrenal response by insulin-evoked hypoglycaemia was not antagonized by losartan (Worck *et al*., 1997).

On the basis of results obtained in cultured rat mesangial cells from both cloning and receptor binding studies, a subclassification of AT_1 receptors was proposed: the receptors which are blocked by low concentrations of losartan (nanomolar) were named AT_{1A} and those which were blocked by low concentrations of PD123319 (nanomolar) were named AT_{1B} (Ernsberger *et al*., 1992; Zhou *et al*., 1993). The first results from functional studies explained on the basis of the existence of two subtypes of AT_1 receptors were those recently published by Cox *et al*. (1995; 1996). These authors observed that in the rat tail artery of the spontaneously hypertensive rats (SHR) there was a synergistic interaction of angiotensin II (0.1 μM) and PD123319 (0.01 – 0.1 μM) leading to an enhancement of noradrenaline release evoked by electrical stimulation and this enhancement was blocked by 0.1 μM losartan. A synergistic interaction was also observed between angiotensin II (0.1 μM) and losartan (0.01 – 0.1 μM) in the rat tail artery of Wistar-Kyoto rats but not in the rat tail artery of SHR. This interaction was prevented by PD123319 (Cox *et al*., 1996). Although having considered that their explanation was not easily reconciled with all the data available, these authors suggested that the synergistic interaction between losartan and angiotensin II might be due either to the unmasking by losartan of a latent population of angiotensin II receptors subserving facilitation of transmitter release, or to the blockade by losartan of an inhibitory action of angiotensin II involving AT_1 receptors which normally opposes its facilitatory action (Cox *et al*., 1996).

In the canine mesenteric and pulmonary arteries in the same concentrations as those used by the authors referred to above losartan did not cause either enhancement or blockade of angiotensin II facilitation of electrically-evoked release of noradrenaline. These different results may depend on the different animal species, on the different concentrations of agonists and antagonists used, on the different technical approaches or on the existence of heterogeneous AT_1 subtypes. In order to rule out at least species' differences as a complicating factor, the present experiments were carried out with two different blood vessels of the dog; the results clearly indicate that the difference found between pre- and postjunctional angiotensin II receptors do not depend on the animal species.

In conclusion, the present results which were obtained by the same methodological approach in two different arteries (mesenteric and pulmonary) of the same species (dog), indicate that: (a) the receptors mediating the prejunctional effects of angiotensin II belong to a subtype that differs from those involved in the postjunctional effects of the agonist; (b) the receptors located at postjunctional level belong to AT_1 subtype, since they are blocked by losartan but not by AT_2 antagonists; (c) prejunctional receptors for angiotensin II apparently belong to neither AT_1 nor AT_2 subtype, since they are not blocked by appropriate concentrations of either AT_1 or AT_2 antagonists.

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